2ORM PTO 1390
(REV 11-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: AMSC-001

U.S. APPLICATION NO. (If known, see 37 CFR 1 5): 09/868753

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATES	PRIORITY DATE CLAIMED
PCT/US00/29231	23 October 2000	22 October 1999

TITLE OF INVENTION: VACCINE COMPOSITIONS

APPLICANT(S) FOR DO/EO/US: John R. MURPHY et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to promptly begin national examination procedures (35 U.S.C. 371 (f)).
4. [ ] The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. [ ] is attached hereto (required only if not transmitted by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. w/PTO-1449, 2 references
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 & 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

-Copy of International Application as published
-Ten (10) Sheets Drawings
-Sequence Listing
-Statement accompanying Sequence Listing
-Diskette Containing Sequence Listing

EXPRESS MAIL LABEL NO. EL807550335US
DATE: June 21, 2001

531 Rec'd PCT/ 21 JUN 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/868753	PCT/US00/29231	AMSC-001

17. [X] The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) – (5)):	
[ ] Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00	
[ ] International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
[ ] International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
[ ] International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
[X] International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	100.00
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	32 - 20 =		x $18.00	216.00
Independent claims	4 - 3 =		x $80.00	80.00
MULTIPLE DEPENDENT CLAIM(s) (if applicable)			+ $270.00	

TOTAL OF ABOVE CALCULATIONS =	396.00
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.	
SUBTOTAL =	396.00
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	
TOTAL NATIONAL FEE =	396.00
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31) + ($40.00 per property).	
TOTAL FEES ENCLOSED =	396.00
* As In Preliminary Amendment	**Amount to be: Refunded**
	Charged

a. [ ] A check in the amount of _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 12-1095 in the amount of $ 396.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to my Deposit Account No. 12-1095 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Lerner, David, Littenberg, Krumholz & Mentlik, LLP
600 South Avenue West
Westfield, NJ 07090
Telephone 908 654-5000

Shawn P. Foley
Signature

SHAWN P. FOLEY
Name

33,071

VACCINE COMPOSITIONS

TECHNICAL FIELD

This invention relates to avirulent or attenuated microbes, their method of preparation, and their use in vaccines.

BACKGROUND ART

Infectious disease remains the largest cause of mortality in the world. A significant proportion of infectious disease associated morbidity and mortality results from bacterial pathogens. One widely applied technique used in controlling the spread and severity of bacterial infection is vaccination. Several notable vaccine examples prevent a number of lethal microbial diseases, the DPT vaccine protects against diphtheria, pertusis and tetanus. The BCG or bacillus Calmette-Guerin vaccine is an example of an attenuated strain which is used worldwide to control the spread of tuberculosis (TB). A central issue in the development of safe an effective bacterial vaccines is the identification of protective antigens or attenuated strains of bacteria which can promote the development of an immune response in the host yet in vaccine composition fail to cause morbidity or martality in the host. A variety of methods can be employed to identify vaccine compositions including the production of attenuated or killed vaccine strains. Often these strains are in single genes which have been shown to play a role in virulence of the organism. Vaccine compositions can include a live or fixed bacterial preparation of the mutant strain, the fixed protein, a fusion protein made of the virulence gene product and a suitable carrier and more recently the DNA encoding the protein.

Attenuated strains offer the potential of presenting a nearly intact complement of pathogen associated antigens to immune system. Furthermore these antigens are likely to be presented in a bacterial context that mirrors that seen during early stages of infection by a virulent strain. This is evidenced by the ongoing practice of using live attenuated strains in the extensive vaccination of both human and livestock. For example BCG for tuberculosis and strain 19 against bovine brucellosis and Sterne's spore vaccine against anthrax in cattle.

The use of live vaccines can present developmental obstacles including the retention of unacceptable levels of virulence, the risk of reversion to

virulence during culture or *in vivo* and lack of efficacy. The ability to create more effective live or attenuated vaccines is in part dependent upon the ability to control and restrict the expression of virulence determinants so as to create vaccine strains that are protective and safe.

Bacteria respond to nutritional stress by the coordinated expression of different genes. This facilitates their survival in different environments. Among these differentially regulated genes are the genes responsible for the expression of virulence determinants. The selective expression of these genes in a sensitive or susceptible host allows for the establishment and maintenance of infection or disease. Virulence include genes which encode toxins, colonization factors and genes required for siderophores production or other factors that promote this process.

The expression of virulence genes in bacteria therefore enable the organism to invade, colonize and initiate an infection in humans and/or animals, however, these genes are not necessarily expressed constantly (constitutively). That is, the bacterium is not always orchestrating gene expression patterns to maximize "infectious" potential. In many circumstances, the expression of virulence genes is controlled by regulatory circuitry which include repressor proteins and a corresponding operon or operator. One class of repressors which is activated upon binding to or forming a complex with a transition metal ion such as iron, zinc or mangenese is thought to control the expression of a subset of genes in a number of Gram positive organisms. When such repressors are activated and associated with virulence gene expression in pathogens, they bind the operator sites thereby preventing production of virulence determinants.

Virulence determinants are most often expressed when the bacterial pathogen is exposed to environmental stress such nutritional restriction. An iron-poor environment is an example of such a condition. In many eucaryotes such an environment is the norm, insufficient iron is present to maintain the repressor in its active state. In the inactive form, the repressor cannot bind to target operators. As a result, virulence genes are de-repressed and the bacterium is able to initiate, establish, promote or maintain infection.

The expression of these virulence determinants is in many bacterial species co-regulated by metal ions. In most instances the metal co-factor that is involved *in vivo* is iron [but can include zinc, nickel, mangenese, cobalts]. In the presence of iron, the repressor is activated and virulence gene expression is halted.

This pattern of gene regulation is illustrated by the following example. The bacterium that causes diphtheria produces one of the most potent toxins known to man. The toxin is only produced under conditions of iron deprivation. In the presence of iron, the bacterial repressor (which in this species is known as diphtheria toxin repressor protein, abbreviated "*DtxR*") binds iron and undergoes conformational changes that activate it and allow it to dimerize and bind a specific DNA sequence called the tox operator. The tox operator is a specific DNA sequence found upstream of the gene that produces the diphtheria toxin, thereby preventing its expression. Typically, during infection of a human host the diphtheria bacillus (or other pathogenic/opportunistic bacteria) grows in an environment that rapidly becomes restricted in several key nutrients. Paramount among these essential nutrients is iron, and when iron becomes limiting the diphtheria bacillus begins to produce the toxin. Moreover, the constellation of virulence genes that *DtxR* controls becomes de-repressed and the diphtheria bacillus becomes better adapted to cause an infection. In the case of diphtheria, the toxin kills host cells thereby releasing required nutrients including iron.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 aligns the amino acid sequences of DtxR, IdeR and SirR.

Figure 2 aligns amino acid sequences of various IdeR/SirR Homologues found in various species of mycobacterium.

Figure 3 aligns and compares the amino acid sequence of various homologues of various DtxR type repressors, including DtxR from *Brevibacterium lactofermentum* (Bl), DtxR from *Corynebacterium diphtheriae* (Cd), IdeR from *Mycobacterium tuberculosis* (Mt), *M. leprae* [P], *M. smegmatis* [P]; DesR from *Streptomyces lividans* (Sl), *M.tuberculosis* SirR, *Staphylococcus aureus* (Sa) SirR, *S. epidermidis* SirR, *Enterococcus fecalis* DtxR homologue [P]. The DtxR homologues from the *Streptococcus gordonii, S. mutans, S. pneumoniae* and *S. pyogenes*. The

consensus amino acid sequences between these members of the DtxR family of iron-dependent repressors is indicated. *, metal ion coordination residues in the Primary site; #, metal ion coordination residues in the Ancillary site; @, the single amino acid residue that interacts with a base in the binding of DtxR dimers to the *tox* operator.

AMS has clones of *M. tuberculosis* IdeR, *S. aureus* and *S. epidermidis* SirR, and DtxR homologues from *Enterococcus fecalis, S. mutans, S. pneumoniae,* and *K. pneumoniae.*

Figure 4: Western blot of cell lysates incubated with polyclonal antibody against DtxR. Lane 1 shows purified DtxR (25.3 kDa). Lanes 3 and 5: lysates from wild type *M.smegmatis* and *M.tuberculosis,* respectively, expressing native IdeR (25.2 kDa.). Lane 4: lysate from the *M.smegmatis* heterodiploid harboring pNBV1/SAD expressing both DtxR(E175K) and IdeR. The molecular weight masses, determined by size standards, are shown at left.

Figure 5: Virulence comparison of wild type *M.tuberculosis* and *M.tuberculosis* DtxR(E175K) mutant. Panel A shows the log CFU of the homogenized spleens of mice sacrificed at 4 week intervals. Panel B shows the log CFU of homogenized lungs at 4 week intervals. Each point represents the mean log CFU of 5-6 mice $\pm$ 1 standard deviation (error bars). Asterisks denote statistically significant differences between groups at a given time point.

Figure 6: Panel A: a 10 week-old representative colony of wild type *M.tuberculosis* (strain CDC1551) on 7H10 agar. Panel B: a 10 week-old representative colony of *M. tuberculosis* DtxR(E175K) on 7H10 agar.

Figure 7: Alignment of the "iron box" consensus sequence, known DtxR binding sites, and putative *M.tuberculosis* DtxR/IdeR binding sites identified by an *in silico* genome search. The "consensus sequence" at the top of the figure represents the compilation of the 9 aligned sequences in the figure. The "published consensus" is drawn from the literature. Gene homologues of the downstream ORFs are shown on the right.

Figure 8: Autoradiographs of gel binding assay between DtxR and putative *M.tuberculosis* DtxR/IdeR binding sites. 100 bp ^{32}P-end-labeled DNA fragments containing *toxO* (lanes 1 & 2), IB-1 (lanes 3 & 4), IB-2 (lanes 5 & 6), IB-

3 (lanes 7 & 8), IB-4 (lanes 9 & 10), IB-5 (lanes 11 & 12) separated in a non-denaturing 6% polyacrylamide gel. Odd numbered lanes contain DNA only ("unbound"), and even numbered lanes contain DNA pre-incubated with purified DtxR ("bound").

Figure 9. Virulence of *S. aureus* is altered in a mouse skin lesion model following 8 days of *in vivo* incubation. 8.0 log CFU of the parent strain MA2181 [RN6390 carrying emtpy shuttle vector pSPT181] and 7.8 log CFU of the complemented MA2004 strain carrying DtxR E175K were injected sub-cutaneously on day 1 abscess size[mm] was measured each day over 8 days and on the last day the abscess was removed and the number of CFU were determined. CFU [**a.**] and abscess size [**b.**] were compared between groups.

SUMMARY OF THE INVENTION

A first aspect of the present invention is directed to a composition containing a virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage. The prokaryote contains a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a dominant, metal ion-independent repressor protein or a partially metal ion independent repressor protein, and a carrier. In preferred embodiments, a promoter is constitutive in nature. In other preferred embodiments, the DNA molecule contains a sequence encoding a metal ion-independent DtxR protein or a partially metal ion-independent DtxR protein. In yet other preferred embodiments, the bacterium is a member of the genus *Mycobacterium, Staphylococcus or Streptococcus*.

The second aspect of the present invention is directed to a method of enhancing protective immunity against infection or disease caused by an opportunistic or virulent prokaryote pathogen in which metal or metal ion-dependent gene regulation confers a growth or an infectious advantage. The method entails administering compositions to the animal. In preferred embodiments, the animal is a human. In other preferred embodiments, the compositions are administered prophylactically *e.g.* prior to the onset of the infection or disease. In yet other

preferred embodiments, the prokaryote contained in the compositions is in a live or killed form.

A relatively aspect directed to is a method of attenuating or reducing the severity of an infection or a disease caused by an opportunistic or virulent prokaryotic pathogen in which metal or metal ion-dependent gene regulation confers a drug or an infectious advantage. The method also entails administering the compositions to the animal, preferably prior to the onset of the infection or disease condition.

Yet another aspect of the present invention is directed to isolated and purified DNA molecules consisting essentially of a sequence encoding a metal or metal ion-independent or a partially metal or metal ion-independent DtxR or homologue thereof. Preferred homologues are IdeR and SirR. In other preferred embodiments, the molecule is placed in an expression cassette or a vector (e.g., a plasmid) so as to be inoperable association with a promoter element, especially a constitutive promoter. Vectors containing the DNA molecules and prokaryotes transformed with DNA molecules (including cultures thereof) are also provided.

A further aspect of the present invention is directed to a method for preparing the compositions. The method entails obtaining a DNA molecule encoding a metal ion-independent repressor protein or a partially metal ion-independent repressor protein. The wild type protein, in its native state, is a metal ion-dependent gene regulator and confers upon a virulent or opportunistic prokaryote a growth or an infectious advantage. The DNA molecule is linked to a promoter, preferably a constitutive promoter, and then introduced into such a virulent or opportunist prokaryote. The DNA is expressed in the prokaryote and inhibits metal ion-dependent gene regulation.

The present invention entails the incorporation of an exogenous DNA encoding a dominant, metal ion (hereinafter "metal") independent or partially metal ion independent mutant repressor into an otherwise virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage. Such prokaryotis include gram-positive and gram-negative bacteria. Without intending to be bound by theory, Applicants believe that the

dominance of the metal-independent mutant repressor subverts the normal patterns of gene regulation (under the control of the native, metal-dependent repressor), thereby creating a recombinant prokaryote that is attenuated or avirulent relative to the wild-type prokaryote. That is, the exogenous mutant repressor renders inoperable or significantly inoperable the normal metal dependent genetic circuitry that occurs *in vivo* and causes in hole or in part the prokaryote carrying such a recombinant genetic compliment to become less infectious or non-pathogenic. This property renders the recombinant prokaryote suitable for use as an immunogen to be formulated into a vaccine.

BEST MODE OF CARRYING OUT THE INVENTION

To prepare the compositions of the present invention, a determination is made as to whether or not the species of interest regulates virulence determinant expression as a function of available metal ion concentrations. This can be done, for example, by screening protein from the bacteria of interest with an antibody for DtxR or other DtxR like proteins to ascertain if a homologous repressor exists in the species. This can also be accomplished using specialized techniques like gel mobility shift assays or the method disclosed in Sun et al, PNAS *95*:14985-14990 (1998), or more common gene expression monitoring methods such northern analysis, and PCR. If the species of interest employs a DtxR type repressor than the expression of this repressor can be elucidated by one of the aforementioned methods and the techniques described here can be employed to build a recombinant attenuated strain for vaccine purposes.

Preferred prokaryotes are Gram positive bacterial species, and particularly those listed below. These species contain DtxR like metal dependent repressors. Specific examples include:

S. pneumoniae	S. agalactia	S.equisimillis
S. meningitis	S. bovis	S.anginosus
S. pyogenes	S. salivarius	S. sanguis
S. suis	S. mutans	Enterococcus faecalis

Staphylococcus species

S. aureus	S. epidermitis

Mycobacteria species

M. tuberculosis	M. avium complex	M. kansasii
M. leprae	M. scrofulaceum	M. fortuitum
M. ulcerans	M. marinum	M. bovis
M. microtii	M. africanum	M. paratuberculosis

Actinomyces species

A. pyogenes	A. israelii	A. bovis
A. viscosus	A. hordeovulneris	A. gerencseriae
A. naeslundii	A. odontolyticus	and others

Listeria monocytogenes

Proprionibacterium acnes

Erysipelothrix rhusiopathiea

The repressor which typifies the class of genetic regulators in the above listed bacteria is the diphtheria toxin repressor DtxR in *C. dihptheriae,* the causitive agent of diphtheria. DtxR is a metal dependent repressor which under limiting concentrations of metal ions becomes inactivated permitting the derepression of a number of virulence genes including diphtheria toxin. This pattern of gene expression is common to both Gram positive and Gram negative bacteria. In Gram positive bacteria, DtxR or DtxR homologues appear to be important metal dependent regulators whereas in Gram negative bacteria, Fur is the significant metal dependent regulator. Some species of pathogen appear to contain both DtxR and Fur like metal dependent repressors. In each case, the presence of repressor bound metal ion is critical for appropriate activity of the repressor which coordinates the repression of gene expression *in vivo*.

The prokaryotes of the present invention having a dominant, metal ion independent or partially metal ion independent mutant of the repressor (such as the diphtheria toxin repressor gene *DtxR* or a *DtxR*-homolog e.g., IdeR, SirR) will render the pathogen unable to effectively establish a full infection. (Hill et al, (1998) Infection and Immunity 66: 4123-4129; and Dussurget et al (1996) Molecular Microbiology 22 536-544; and Pohl et al (1999) J. Molecular Biology 285 1145-1156). Apart from the targeting vector sequences or plasmid DNA used to generate

the attenuated strains and the mutant repressor gene, the recombinant prokaryote is in all other aspects identical genetically to the wild-type organism. The presence of the dominant iron independent repressor results in a phenotypic change in the organisms virulence.

In preferred embodiments, the exogenous DNA comprises a sequence encoding a dominant, metal-independent DtxR or a functional fragment, variant or homologue thereof (collectively referred to as "a DtxR protein"). DtxR is a metal iron-dependent DNA-binding protein having a deduced molecular weight of 25,316 and which functions as a global regulatory element for a variety of genes on the *C. diphtheriae* chromosome. See Tao *et al.*, Proc. Natl. Acad. Sci. USA *89*:5897-5901 (1992); Schmitt *et al.*, Infect. Immun. *59*:1899-1904 (1994). For example, *DtxR* regulates the expression of the diphtheria toxin structural gene (*tox*) in a family of closely related Corynebacteriophages. The repressor has also been shown to regulate a number of other iron dependent genes. The gene for DtxR and a number of DtxR homologues have been cloned and sequenced. A number of detailed structural and functional studies have be performed to analyse DtxR. See Boyd *et al.*, Proc. Natl. Acad. Sci. USA *87*:5968-5972 (1990); Schmitt *et al.*, *supra*. *DtxR* is activated by divalent transition metal ions (*e.g.*, iron). Once activated, it specifically binds the diphtheria *tox* operator and other related palindromic DNA targets. See Ding *et al.*, Nature Struct. Biol. *3(4)*:382-387 (1996); Schiering *et al.* Proc. Natl. Acad. Sci. USA *92*:9843-9850 (1995); White *et al.*, Nature *394*:502-506 (1998). DNA sequences encoding DtxR from various *C. diphtheria* strains are defined by accession numbers M80336,M80337, M80338, and M34239.

Functional fragments or variants of *DtxR*, when activated, retain their binding activity to the *tox* operator (or a functional fragment thereof) and/or the *DtxR* consensus binding sequence. *DtxR* fragments and variants can be identified by standard techniques such as mutagenesis. Tao *et al.*, Proc. Natl. Acad. Sci. USA *90*:8524-8528 (1993) identified important residues for DtxR function and analysis. Other variants are disclosed in Tao *et al.*, Mol. Microb. *14(2)*:191-197 (1994). *Tao* discloses that some *DtxR* alleles have different amino acid sequences, e.g., the *DtxR* allele from strain 1030(-) of *C. diphtheriae* was found to carry six amino acid

substitutions in the C-terminal region, none of which affected the iron-dependent regulatory activity of *DtxR* (1030) (Tao II). See also Boyd *et al.*, J. Bacteriol. *174*:1268-1272 (1992) and Schmitt *et al.*, Infect. Immun. *59*:3903-3908 (1991). Thus, DtxR fragments and variants may be mutagenized to an iron-independent genotype.

Many other bacterial species employ regulatory circuits and repressor proteins that exhibit high degrees of sequence similarity to *DtxR*. (Posey et al (1999) Proc Natl Acad Sci 96 10887-10892, Que and Helmann (2000) Molecular Microbiology 35 1454-1468, Kitten et al, (2000) Infect and Immun 68 4441-4451, Manabe (1999) Proc Natl Acad Sci 96 12844-12848. Thus, dominant, metal-independent *DtxR* homologues may also be employed in the methods of the present invention. Iron dependent regulator (*IdeR*), isolated from *Mycobacterium tuberculosis*, has been found to share 60% amino acid homology with *DtxR*. See Schmitt *et al.*, Infect Immun. *63(11)*:4284-4289 (1995). See also Doukhan *et al.*, Gene *165(1)*:67-70 (1995), which reports and references *DtxR* homologs in *Mycobacterium smegmatis* and *Mycobacterium leprae*. *DtxR* homologues have been cloned in other gram-positive organisms including *Brevibacterium lactofermentum* and *Streptomyces lividans*. See Oguiza *et al.*, J. Bacteriol. *177(2)*:465-467 (1995); Günter *et al.*, J. Bacteriol. *175*:3295-3302 (1993); and *Schmitt et al.*, Infect. Immun. *63*:4284-4289 (1995). Staphylococcal iron regulated repressor (SirR), native to *Staphylococcus epidermitis*, is another known DtxR homologue. These proteins bear a common feature they share a remarkably high sequence similarity in the respective N-terminal 139 amino acid regions, especially those amino acids involved in DNA recognition and transition metal ion coordination.

A collection of accession numbers for sequences that are either homologous to *DtxR* or contain a consensus tox O/P is presented in Table 1. See http://www.ncbi.nlm.nih.gov/BLAST and http://www.ncbi.nlm.nih.gov/unfinishedgenomes.html. See also, Altschul, *et al.*, J. Mol. Biol. *215*:403-410 (1990); Gish, *et al.*, Nature Genet. *3*:266-272 (1993); Madden, *et al.*, Meth. Enzymol. *266*:131-141 (1996); Altschul, *et al.*, Nucleic Acids Res. *25*:3389-3402 (1997); and Zhang, *et al.*, Genome Res. *7*:649-656 (1997). This

high degree of sequence similarity and homology is indicative of a widely conserved metal ion dependent regulatory pathway employing *DtxR*-family repressors. It is noteworthy that many important human and animal pathogens are present in this collection of bacteria. Dominant metal independent repressors can be generated and introduced inot a prokaryote. Such mutants alter virulent phenotype *in vivo* and may be used asviaccines.

Table 1

***DtxR* Homologs and Species with DtxR Binding Sites**

	Pathogenic Human/Veterintary Applications		Other	
	CAA67572	S. epidermitis	L35906	C. glutamicum
	Gi 1777937	T. pallidum	Z50048	S. pilosus
	CAA15583	M. tuberculosis	Z50049	S. lividans
	U14191	M. tuberculosis		
	L78826	M. leprae		
	M80336	C. diphtheriae		
	M80337	C. diphtheriae		
	M34239	C. diphtheriae		
	M80338	C. diphtheriae		
	AAD18491	C. pneumoniae		
	Gi 3328463	C. trachomatis		
	TIGR 1280	S. aureus		
	OUACGT	S. pyogenes		
	Sanger 518	**B. bronchoseptica**		
	Sanger 1765	M. bovis		
	Sanger 520	B. pertusis		
	WUGSC	K. pneumoniea		
	TIGR 1351	E. faecalis		
	AE000783	B. burgdorferi		
	TIGR1313	S. pneumoniea		
	Snager 632	Y. pestis		
	AE001439	H. pylori		
	TIGR 1752	V. cholera		
	TIGR1097	**C. tepidum**		
	U14190	M. smegmatis		
	Gi 2621260	M.thermoautotrophicum		
	Gi 2622034	M. thermoautotrophicum		
	M50379	M. jannaschi		
	Q57988	M. jannaschi		
	O33812	S. xylosus		
	Gi 264870	A fulgidus		
	Gi 2648555	A fulgidu		
	Gi 2650396	A fulgidus		
	Gi2650706	A fulgidus		
	BAA79503	A. pernix		
	CAB49983.1	P. abyssi		
	BAA30263	P. horikoshi		
	AL109974	S. coelicolor		
	L35906	B. lactofermentumStanford 382 **S. meliloti**		
	TIGR 76	**C. crescentus**		
	TIGR 24	**S. putrificacieus**		
	AE000657	**A. aeolius**		
	TIGR 920	**T. ferrooxidans**		

Preferred gram positive pathogenic bacteria include *Mycobacterium bovis, Mycobacterium leprae, Mycobacterium paratuberculosis, Mycobacterium tuberculosis, Mycobacterium avium, Staphylococccus aureus, Staphylococcus epidermitis, Streptoccus mutans and Streptococcus pneumoniae*. The preferred dominant DtxR (or homologue) repressors are metal independent and contain a single amino acid change to convert the native, metal-dependent repressor to metal independent regulation.

A preferred metal independent repressor is the mutant E175K DtxR. This same substitution can be made in DtxR fragments and variants. In addition, DtxR homologues that exist in other bacterial species may be mutated at the corresponding position. Figure 1 displays the degree of amino acid homology between DtxR and to homologous proteins, IdeR from *Mycobacterium tuberculosis* and SirR from *Staphylococcus epidermitis*. As shown in fig. 1, the single amino acid at residue E175K in DtxR, the glutamic acid is conserved in IdeR hence a 177K mutation of IdeR would most likely have the analogous functional implications. Dussurget et al (1996) Molecular Microbiology 22 536-544 and Pohl et al(1999) J. Molecular Biology 285 1145-1156).

Figure 2 presents a comparison of IdeR/SirR homologues found in other mycobacterium that cause significant disease including; *M. tuberculosis, M smegmatis, M. leprae* and a SirR clones from *M. tuberculosis*. Shown in bold is the conserved glutamic acid in the C-terminal region of these repressors that can potentially be mutated to yield an iron independent version of each of these repressors. Thus, corresponding mutations would be expected to result in a metal-independent genotype. Figure 3 illustrates the amino acid sequence homology of a number of homologous DtxR type repressors. These repressors differ slightly in their sequence length and the ClustalW program used to carry out a logical alignment adjusts for these differences. See NCBI [www.ncbi.nlm.nih.gov] web site and Baylor College of Medicine Search Launcher[http//.gc.bcm.tmc.edy] for details. Indicated by Bold 'E' is the highly conserved glutamic acid residue in the C-terminus of these repressors which is a possible target for generating metal ion independent versions of each of these represssors.

A combination of standard techniques may be used to make other dominant, metal-independent DtxR proteins, namely mutagenesis followed by tests to determine if a given mutant binds the corresponding operator. A number of theoretical mutations can be incorporated which will convert the repressor to be metal-independent. To generate repressor DNA clones with a random distribution of mutated bases, any of several saturation mutagenesis techniques can be utilized. DNA, plasmid preparation, and DNA sequence analysis are performed according to standard methods. The saturation of random mutations throughout the length of repressors DNA of interest introduces random changes in amino acid sequence throughout the encoded protein that potentially can confer a metal independent phenotype on the mutant repressor.

Several approaches can be pursued to generate effective vaccines relying upon DtxR. In preferred embodiments, DtxR homologues are identified in a species of interest. This can be achieved by performing PCR on genomic DNA from the species of interest using primer sets compatible to conserved domains within the DtxR family. Specific sequences may be composed of degenerate primers flanking the iron binding domain, or the helix-turn-helix domain. Cloning may also be performed from phage libraries using DtxR conserved regions as probes, utilizing the PSTD system or by *in silico* searches and de novo *in vitro* synthesis.

The DtxR has a domain structure which is composed of a helix-turn-helix domain, and N-terminal iron binding domain. There are a number of additional highly conserved sequences including a proline rich region which lies between the HLH and C-terminal domain. The polymerase chain reaction allows the amplification of any DNA target from a population of DNA molecules if compatible oligonucleotide primers can be identified. While a number of software programs are available to assist in the design of effective primers, optimal primer sets often require empirical determination.

Cloning of DtxR homologues from any given species can be achieved by mixing primers with genomic DNA isolated from the species of interest in the appropriate ratios in the presence of free oligonucleotides, optimized buffer conditions and the TAQ polymerase (or a suitable version of a thermostable

polymerase) and cycling the reaction conditions with the aid of an automated thermocycler. The PCR reaction generates a series of products that represent target DNA sequences that are bounded by sequences homologous to the selected 5' and 3' primers. Thus, the selection of primers which lie within conserved regions of DtxR will likely bind under the appropriate conditions to homologous DNA that will likely represent a gene or domain similar to that of DtxR.

DtxR homologues may further be identified by screening genetic libraries of a given species created in *E. coli*. Pathogen libraries can then be screened by radio-labeled probes generated from DtxR clones, oligonucleotides from the DtxR sequence, or protein assays using antibodies directed against DtxR.

Sun, *et al.* have developed a screen can be used as a positive selection assay for DtxR homologues. See Sun, *et al.*, PNAS 95:14985-14590 (1998). This screen requires only the generation of a gDNA library from the species of interest be transformed into the appropriate background host. Colonies which appear upon selection by chloramphenicol can only arise if a functional DtxR like protein is being expressed from the cloned gDNA fragment. Once a clone has been identified, sequencing and sequence analysis will reveal a gene which has at least partial sequence homology to DtxR and fucntional equivalence based on the PSDT screen developed by Sun *et al.*

Once identified, these clones become the substrates from which a vaccine strain is assembled. Two approaches are preferred. The first approach is a knock–in approach which is coupled with *in vitro* mutagenesis or PCR mutagenesis. The knock-in approach is focused on generating strains having dominant activated repressors. These strains contain a defective or altered copy of DtxR or a DtxR homologue that containing at least one but up to several mutations resulting in a repressor that recognizes and binds the toxAPO or suitable cognate binding sites in both the presence and absence of iron (or the appropriate metal ion). These metal ion independent mutants are cloned into suitable vectors (e.g., targeting vectors having a selectable marker and restriction sites) to develop strains expressing dominant activated DtxR constitutively. These plasmids are used to generate knock-in vaccine strains, essentially altered forms of the wild-type virulent strain differing in only the

presence of a dominant activated DtxR or DtxR-homologous repressor. The constructs can also be used in gene replacement strategies in which the mutant metal independent repressor gene replaces the endogenous wild type gene. (Howard et al (1995) Gene 166 181-182, Jacobs et al (1991) Mthds in Enz 204 537-555., Rubin et al (1999) Proc Natl Acad Sci 1644-1650), Caparon and Scott (1991) Mthds in Enz 204 556-586, Norgren, Caparon and Scott, (1989) Infect and Immun 57 3846-3850, Biswas et al, (1993) J Bact. 175 3625-3635. Such a strain is effectively avirulent since its ability to up-regulate iron dependent virulence genes has been crippled.

Metal ion independent clones can be sequenced to identify the specific amino acid changes that confer the iron independent phenotype. Again, a preferred mutant is characterized by a change from glutamic acid to lysine at position 175 in native DtxR. In general, however, multiple mutations are also embraced by the present invention. Multiple mutations are advantageous because they greatly decrease the likelihood of reversion to wild-type function. This likelihood becomes statistically more akin to that of a strain which has all of its iron regulated genes knocked out. Multiple mutations either clustered or distributed through out the repressor can result in the same phenotype. For example, a double mutant DtxR with the replacement of asparagine at position 130 with glycine and the glutamine at position 181 with arginine was also identified with a metal independent phenotype. In addition a mutant with an intermediate phenotype was also identified having a total of six mutations[valine 5 to isoleucine, aspartic acid 110 to glutamic acid, valine 112 to phenylalanine, isoleucine 153 to threonine, aspartic acid 197 to glutamic acid, threonine 220 to alanine]. These mutations produced a partially metal dependent phenotype. Thus, the present invention also embraces the use of partially metal ion-independent repressors. It is believed that expression of these repressors allow the partial colonization of the host which promotes the development of a robust protective immune response. The metal dependent phenotype can be readily revealed by linking a readily assayed reporter gene to the *tox O/P* in *E. coli* and a assessing reporter function under metal and metal conditions as described by Sun, *et al.*

More generally, however, the DtxR mutants of the present invention are not limited to those characterized by single or multiple amino acid substitutions.

Insertion and deletion mutants are also contemplated. These mutants may be identified using the instantly disclosed techniques as well.

Strains and putative mutants can be tested for metal independent phenotype by several approaches including the PSTD screen described in Sun *et al.* Additional methods of determining if conversion to iron independent phenotype include computer modeling, structural analysis, dimerization analysis by gel electrophoresis, DNA binding by electrophoretic mobility shift assay, transcriptional profiling, tissue culture and in vivo virulence assays. Ultimately, *in vivo* screening will be required to determine if the iron independent phenotype is stable in a host environment and to determine if virulence is attenuated. Attenuated virulence can be defined by biochemical, physiological and immunological markers but will minimally include an assessment of ED_{50} or LD_{50} in wild-type, knock-in, replacement and recombinant strains.

A second rational mutagenesis strategy can be utilized to generate metal-independent mutants. This strategy relies upon the conserved domain structure of the DtxR family of repressors. Several DtxR iron independent mutants have been identified and published. These mutants define two classes of iron independent mutations which are likely to alter iron dependent regulation in DtxR homologues. Site directed mutagenesis of the analogous amino acid residues in DtxR homologues may have the same iron independent phenotype. In addition as the data from structural studies grows it will likely be possible to construct mutants that replace amino acids involved in the coordination of iron binding or dimerization that result in an iron independent phenotype.

Mutagenesis is then carried out to generate a library of the desired mutants of this DtxR homologue of interest. Identification of iron independent mutants from this population is achieved by using the PSTD system. Using the cloned and mutant homologue genes, one can select for the growth of colonies in the PSTD strains in the presence of dipyridyl (DP). Dipyridyl chelates iron from the media and therefore leads to the disassociation of DtxR homologues from the regulatory regions of the genetic elements in the PSTD screen. As a result, all iron

dependent repressors will not be able to survive chloramphenicol selection, in contrast iron independent mutants will grow.

Mutants of DtxR are generated in accordance with standard techniques. Polymerase chain reaction (PCR) mutagenesis of the *dtx*R gene is described in Vartanian *et al.* [Vartanian, J.-P., Henry, S., & Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions. *Nucleic Acid Res.*, 24, 2627-2631.]. Briefly, *Bgl*II-tagged primers 1515 (5'-ACCAGATCTGCCGAAAAACTTCGA-3') and 1516 (5'-ACCAGATCTCCGCCTTTAGTATTTA-3') were used to PCR amplify *dtx*R from plasmid pRDA which carries the wild type *dtx*R operon. The products of the amplification were then digested with *Bgl*II and ligated either into *Bgl*II-linearized pSC6M1 and transformed into *E. coli* TOP10/λRS65T, or ligated into *Bam*HI digested pBR322 and transformed into *E. coli* TOP10/λRS65T/pSC6. Iron-independent mutants of DtxR were then selected on LB agar plates supplemented with Cm and DP in accordance with the procedure described in Sun, *et al.*

Broadly speaking, these mutations should conserve the structural integrity and maintain the ability of the repressor to bind and repress gene expression through the consensus or near consensus *tox* P/O sites. Bacterial clones containing mutagenized *dtxR* can be analysed by DNA sequencing and used in functional biochemical assays such as electrophoeretic mobility shift assay, native gel analysis and gluteraldehyde crosslinking studies to reveal the activated state of the repressor in question under metal limiting conditions. This can be determined through gel shift analysis or by functional assays, but it is preferably made using the one-step method described by `Frigg`, et al [Sun, L., vanderSpek, J. & Murphy, J. R. (1998)*Proc. Natl. Acad. Sci. USA,* 95, 14985-14990]. For gel shift analysis the native *tox* operator (i.e., 5'-ATAATTAGGATAGCTTTACCTAATTAT-3') is a 27 base pair interrupted palindromic sequence upstream of the diphtheria *tox* structural gene can be used as a probe. This sequence features a 9-base pair inverted repeat sequence that is separated by 9 base pairs. See Kaczorek *et al.*, Science *221*:855-858 (1983); Greenfield *et al.*, Proc. Natl. Acad. Sci. USA *80*:6853-6857 (1983); Ratti *et al.*, Nucleic Acids Res. *11*:6589-6595 (1983); and Fourel *et al.*, Infect. Immunol.

57:3221-3225 (1989). It overlaps both the -10 region of the *tox* promoter and the transcriptional start sites at -45, -40 and -39 upstream of the diphtheria toxin structural gene. See Boyd *et al.*, J. Bacteriol. *170*:5940-5952 (1988). The minimal essential DNA target site, i.e., 5'-GTAGGTTAGGCTAACCTAT-3', is a 19 base pair sequence that forms a perfect palindrome around a central C or G -- is described in Tao and Murphy, Proc. Natl. Acad. Sci. USA *91*:9646-9650 (1994). Additional probes are variants of ToxO based on the *DtxR* consensus-binding sequence (5'-ANANTTAGGNTAGNCTANNCTNNNN-3'). Variants are defined by the following sequence: 5'-TWAGGTTAGSCTAACCTWA-3'. Thus the function of the repressor and mutant can be defined by recognition and binding or regulation of gene expression via the sequences or variants described above.

Once a dominant, metal-independent DtxR DNA clone is identified, it can be produced and manipulated in accordance with techniques known in the art. For example, they may be generated using standard chemical synthesis techniques. See, *e.g.*, Merrifield, Science *233*:341-347 (1986) and Atherton *et al.*, Solid Phase Synthesis, A Practical Approach, IRL Press, Oxford (1989). Preferably, they are obtained by recombinant techniques. Standard recombinant procedures are described in Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second ed., Cold Spring Harbor, New York, and Ausubel *et al.*, (eds.) Current Protocols in Molecular Biology, Green/Wiley, New York (1987 and periodic supplements). The appropriate sequences can be obtained from either genomic or cDNA libraries using standard techniques. DNA constructs encoding the DNA gene segments may also be prepared synthetically by established methods, *e.g.*, in an automatic DNA synthesizer, and then purified, annealed, ligated and cloned into suitable vectors. Atherton *et al.*, *supra*. Polymerase chain reaction (PCR) techniques can also be used. See *e.g.*, PCR Protocols: A Guide to Methods and Applications, 1990, Innis *et al.* (ed.), Academic Press, New York.

The DNA encoding the mutant metal-independent repressor is operably linked to a promoter element. Preferred promoters include the endogenous DtxR promoter or the promoter of the DtxR homologue in question or any suitable constitutive promoter functional in the species of interest. the constructs may further

contain an associated selectable marker gene to follow maintenance of the mutant construct. For example in Mycobacterial species a hygromycin resistance gene in an *E. coli* shuttle vector provides great utility for cloning and expression of a mutant IdeR or another dominant metal ion independent repressor [DtxR E175K]. [Bishai *et al* Gene 1995 166:181-182] In other embodiments, the vectors also contain a copy of a gene lethal to the bacterium under the control of the metal dependent regulator. Examples of such genes include antibiotic genes, restriction enzymes, proteolytic enzymes, lethal phage genes or any gene whose product once expressed would kill the bacterium. The presence of this "suicide cassette" further ensures that vaccines containing the prokaryote in live form do not revert to any significant degree and cause disease *in vivo*.

Suitable cloning and expression vectors are readily available from a number of sources. In preferred embodiments, the construct is introduced into the prokaryote by way of a vector, in which case the construct may be formed prior to or upon introduction of the DNA into the vector. A vector for a given species must contain an origin of replication, a selectable marker and a functional promoter by which the mutant metal ion independent repressor can be expressed in the strain of interest. (Howard et al (1995) Gene 166 181-182, Jacobs et al (1991) Mthds in Enz 204 537-555., Rubin et al (1999) Proc Natl Acad Sci 1644-1650), Caparon and Scott (1991) Mthds in Enz 204 556-586, Norgren, Caparon and Scott, (1989) Infect and Immun 57 3846-3850, Biswas et al, (1993) J Bact. 175 3625-3635.

The vectors are introduced into the cells in accordance with standard techniques such as transformation, co-transformation, direct transfection (*e.g.*, mediated by calcium phosphate or DEAE-dextran) biolistics and electroporation. The recombinant cells are then cultured via standard techniques. Conditions may vary depending upon the prokaryote (e.g., bacterial species) being used. In general, culturing is continued from about 24 to 48 hours at a temperature between about 30 and about 39°C, preferably 37°C. The recombinant cells are cultured in an appropriate complete medium containing a selectable marker to assure a pure population. The strains can also be counter engineered as described below so that if significant levels of the metal independent repressor is not produced a suicide gene

will be de-repressed resulting in the death of the vaccine strain *in situ*. Iron is an essential element for both the bacterial pathogen and its animal host; thus, successful competition for this element is an essential component of the infectious process. The concentration of free iron in the mammalian host available to an invading bacterial pathogen is also extremely limited. As a result, the expression of virulence determinants (*e.g.*, colonization factors, siderophores, hemolysins and toxins) by bacterial pathogens is regulated by iron. Accordingly, the vaccine cultures must be propageted in a complete medium containing iron and other divalent metal cations to facilitate their proliferation.

The vaccines of the present invention may be used in a wide variety of vertebrates, particularly man and domestic animals such as bovine, ovine, porcine, equine, caprine, domestic fowl, *Leporidate*, or other animals that may be held in captivity or may be a vector for a disease affecting a domestic vertebrate.

Pathogens of interest include any specie of microorganism which causes disease and relies entirely or partially upon a repressor mediated regulation of metal-dependent virulence. The present invention relates to methods of vaccinating a host with live recombinant bacteria to elicit protective immunity in the host. The recombinant vaccine can be used to produce humoral antibody immunity, cellular immunity (including helper and cytotoxic immunity) and/or mucosal or secretory immunity.

The manner of application of a vaccine strain may be varied widely, any of the conventional methods for administering an attenuated vaccine being applicable. These include aerosol applications, oral applications, in drinking water, on a solid physiologically acceptable base, or in a physiologically acceptable dispersion, parenterally (e.g., subcutaneously, intramuscularly, intravascularly or intraperitoneally), by injection, by *in ovo* inoculation or the like. The dosage of the vaccine (e.g., number of prokaryotic cells, number of administrations, period of administration, etc.) will vary according to the vaccine strain used and the species, age, and size of host to be protected. Persons skilled in the art will be able to determine the doseage to be administered so as to provide a sufficient immune

response. The recombinant prokaryotes in the composition may be "live" or in "killed" form, as these terms are commonly used in the vaccine art.

The formulation of vaccine strain compositions may also vary widely. Pharmaceutically acceptable vehicles such as water are expected to be useful for oral administration. Other such vehicles including normal saline may be used for parenteral, cloacal or other routes of administration. The vaccine compositions may also be admixed with food for some applications.

The following example is not intended to limit the scope of the invention in any manner.

Example 1

Attenuation of Virulence in *Mycobacterium tuberculosis* Expressing a Constitutively Active Iron Repressor

This example describes the construction of a candidate strain which is severely attenuated with respect to the parent wild type strain yet which persists long enough to allow the host to mount an immune response.

Summary

With over one-third of the world's population latently infected with *Mycobacterium tuberculosis,* the global burden of tuberculosis is staggering. The emergence of multi-drug resistant strains and the increased susceptibility of the HIV-infected further highlights the need for elucidation of the molecular pathogenesis of *M.tuberculosis* and its virulence genes.

Iron plays a critical role in the regulation of virulence of many bacterial pathogens. (1) In tuberculosis, there is indirect clinical and *in vitro* evidence that iron regulation is important to the virulence of this microbial pathogen. (2-5) Iron is an essential nutrient for the survival of most organisms and has played a central role in the virulence of many infectious disease pathogens. Mycobacterial IdeR is an iron-dependent repressor that shows 80% identity in the functional domains with its corynebacterial homologue, DtxR. In a novel approach to attenuation, *Mycobacterium tuberculosis* has been transformed with a vector expressing an iron-independent, positive dominant, corynebacterial *dtxR* hyperrepressor, DtxR(E175K). Western blots of whole cell lysates of *M.*

tuberculosis expressing the *dtxR*(E175K) gene revealed the stable expression of the mutant protein in mycobacteria. BALB/c mice were infected by tail vein injection with 2x 10^5 organisms of wild type or *M.tuberculosis* transformed with the *dtxR* mutant. At 16 weeks, there was a 1.2 log reduction in bacterial survivors in both spleen ($p=0.0002$) and lungs ($p=0.006$) with *M.tuberculosis* DtxR(E175K). A phenotypic difference in colonial morphology between the two strains was also noted. A computerized search of the *M.tuberculosis* genome for the palindromic consensus sequence to which DtxR and IdeR bind, revealed six putative "iron boxes" within 200 base pairs of an open reading frame. Using a gel shift assay, it was shown that purified DtxR binds to the operator region of five of these. Attenuation of *Mycobacterium tuberculosis* can be achieved by the insertion of a plasmid containing a constitutively active, iron-insensitive repressor, DtxR(E175K), which is a homologue of IdeR. The results demonstrate that IdeR controls genes essential for virulence in *M.tuberculosis*.

In a phylogenetically related organism, *Corynebacterium diphtheriae,* iron depletion results in the derepression of virulence genes such as the diphtheria toxin *(tox)* gene by DtxR (diphtheria toxin repressor). The corynebacterial DtxR has a homologue in *M.tuberculosis,* IdeR (iron-dependent repressor). In the amino terminal 140 amino acids that contain the Fe^{2+} and DNA-binding domains of DtxR, IdeR shares 80% identity with DtxR. (6) In 1995, *ideR* was first described by Doukhan *et al.* in conjunction with the *sigA sigB* cluster of genes. (7) Subsequently, the ability of mycobacterial IdeR to bind to the corynebacterial *tox* operator region in a metal ion-dependent manner was demonstrated by gel shift assay. (8) Mutation of *ideR* in *M.smegmatis* resulted in derepressed siderophore production in high iron conditions. (9) These findings parallel those described in corynebacterial *dtxR* and suggest that the homology between these two genes may allow for cross-genus functional complementation.

Using a positive genetic selection system to clone *dtxR* alleles, Sun *et al.* isolated and characterized a series of DtxR mutants created by PCR mutagenesis. (10) One of the mutants which bound to the *tox* operator (*toxO*) and constitutively repressed reporter gene expression in an iron-independent manner was characterized

and found to have a single amino acid substitution of lysine for glutamic acid at position 175 (DtxR(E175K)). In merodiploid strains harboring both wild type *dtxR* and mutant *dtxR*(E175K) genes, Sun *et al.* found the mutant to be dominant over the wild type allele.

Methods

Strains, Plasmids, and Cultures The bacterial strains and plasmids used in this study are listed in Table 2. (10-14) *Escherichia coli* cultures were grown in Luria broth or Luria agar supplemented with ampicillin (100g/ml) or hygromycin (200g/ml). *M.tuberculosis* CDC 1551 and *M.smegmatis* cultures were grown in standard Middlebrook 7H9 broth (Difco), supplemented with albumin dextrose complex (ADC), 0.1% glycerol, and 0.05% Tween 80 at 37°C in roller bottles. (15)

Construction of *dtxR* (E175K) Shuttle Vector Plasmid A 1.5 kb *Bam*HI-*Hin*dIII fragment of DNA from pSDM2 was cloned into pNBV1. The resulting recombinant plasmid, pNBV1/SAD was cloned in *E.coli* DH5 and purified using the Qiagen system (Qiagen, Chatsworth, CA). (16) Purified plasmids were then electroporated into *M.tuberculosis* CDC1551 by standard protocols. (15)

Western Blot Analysis Recombinant *E.coli* and mycobacteria were lysed in 3M urea, 0.5% Triton X-100, 3.25M DTT, 2% Pharmalyte (Pharmacia Biotech, Piscataway, NJ), PMSF (100g/ml) and leupeptin (2g/ml). Using 0.1 mm glass beads, the samples were homogenized twice in a Mini-bead-beater (Biospec Products, Bartlesville, OK) at maximum speed for 1 minute. Samples were centrifuged to remove cellular debris and unlysed cells. After separation by 12% SDS polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, UK) by semi-dry technique (Transblot SD, Hercules, CA) and blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (PBS-T) for 1 hour. Membranes were then incubated overnight in PBS-T with rabbit anti-DtxR polyclonal antibodies at the appropriate concentration at 4°C. (17) After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted in PBS-T for 2 hours. The Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) was used for autoradiograph development.

Murine Tuberculosis Infection Model 6-8 week-old BALB/c mice were infected by tail vein injection with $2x10^5$ organisms of wild type or *M.tuberculosis* DtxR(E175K). Bacterial infection was monitored over a 119-day period. Colony forming units (CFU) in spleen and lungs were assessed at 4 week intervals by serial dilutions of organ homogenates plated on 7H10 Middlebrook agar containing cycloheximide (50 g/ml), carbenicillin (50 g/ml), trimethoprim (20 g/ml), and polymyxin (200 units/ml). (18)

DNA Gel Shift Binding Assay The DNA migration retardation assay was performed as previously described. (19) Purified DtxR protein was isolated by methods as described. (20) Radiolabeled DNA iron box fragments were generated by PCR using 100 ng of ^{32}P-end-labeled primer mixed with 150 ng of unlabeled primer and template DNA from gel-purified 100 bp cold fragments containing the iron box of interest. Binding reactions were carried out in 10mM Tris-OAc (pH7.4), 1mM EDTA, 50mM KCl, 1mM DTT, 5% glycerol, 50g/ml calf thymus DNA. Binding reactions were equilibrated for 30 minutes and then loaded onto a non-denaturing 6% acrylamide gel. (21)

Results

Expression of the corynebacterial *dtxR* gene in mycobacteria

The 1.5 kb corynebacterial DNA fragment cloned in pNBV1/SAD contained 500 bp of 5' non-coding sequences as well as the entire *dtxR*(E175K) open-reading frame. To determine if the corynebacterial mutant *dtxR*(E175K) gene was expressed in mycobacteria, we transformed *M.smegmatis*, a fast growing strain of mycobacteria, with pNBV1/SAD. Whole cell lysates prepared from *M.smegmatis* cultures were separated by 12% SDS PAGE. Figure 4 shows a Western blot developed with polyclonal anti-DtxR antibodies. As illustrated, these antibodies recognize both DtxR and IdeR because of their significant antigenic similarity. Although the deduced molecular mass of IdeR (25.2kDa) differs by only 0.1kDa from DtxR (25.3kDa) we have repeatedly observed anomalous accelerated migration of IdeR in our SDS-PAGE gels in which it runs at 23kDa in spite of its mass of 25kDa. This phenomenon has also been noted by Schmitt *et al.* (8) In preparations from *M.smegmatis* harboring pNBV1/SAD (Fig4, lane 4), two distinct bands appear.

Because *dtxR*(E175K) is expressed from a multicopy plasmid, significantly more DtxR(E175K) protein is made than the chromosomally expressed IdeR. Similar results in *M.tuberculosis* transformed with pNBV1/SAD were also found (results not shown). The *in vitro* growth rate of wild type *M. tuberculosis* was indistinguishable from that of *M. tuberculosis* DtxR(E175K) by the BACTEC radiometric growth monitoring system.

Attenuation of Virulence in *M.tuberculosis* Expressing the Constitutively Active DtxR Hyperrepressor After confirming that the corynebacterial mutant *dtxR* was expressed in transformed mycobacteria, we turned to an *in vivo* animal model to test the effect of the hyperrepressor on virulence. Forty-eight BALB/c mice were inoculated with 2 x 10^5 CFU of CDC1551 *M.tuberculosis* or *M.tuberculosis* DtxR(E175K) by tail-vein injection. Both animal weights and the tissue burden of surviving bacteria were monitored over time. Mice infected with wild type *M.tuberculosis* began to lose weight beginning at 13 weeks while the *M.tuberculosis* DtxR(E175K)-infected animals initially gained weight, then maintained stable weights for the duration of the experiment. At 17 weeks, there was a statistically significant difference of 1.7 gms (p=0.006 by two-tailed t-test) between the wild type and DtxR(E175K) groups.

Figures 5A and 5B show the survival of the two *M.tuberculosis* strains in lungs and spleens of mice over time. At 17 weeks, there was a 1.2 log attenuation in virulence of the DtxR(E175K) expressing strain compared with wild type which was statistically significant in both spleen(p=0.0002) and lungs (p=0.006). Analysis of the colonies from the mouse tissues at 12 weeks showed that 99% of the colonies were hygromycin resistant indicating maintenance of the pNBV1/SAD plasmid. Histopathologic inspection of spleen and lungs of wild type and DtxR(E175K) expressing strains corroborated our CFU data with fewer visible acid fast bacilli at 17 weeks in histologic sections of mouse organs from animals infected with the *M.tuberculosis* DtxR(E175K) than with the wild type.

Differences in Colonial Morphology Between Strains Colonies of *M.tuberculosis* DtxR(E175K) grown from frozen stocks on 7H10 Middlebrook agar showed no difference in growth rate *in vitro* as compared to wild type CDC1551, but

were noted to have a distinct colonial morphology (see figures 6A and 6B). The recombinant strain colonies were rougher and drier-appearing and were more raised and wrinkled than wild type colonies. In addition, yellow pigmentation was also noted in the DtxR(E175K) expressor. Both strains exhibited a spreading phenotype and were crenelated at the periphery.

Identification of Iron Boxes An imperfect palindromic consensus sequence of the "iron box" for DtxR/IdeR has been established by *in vivo* and *in vitro* methods. (8, 22, 23) This consensus sequence is listed at the top of Figure 7. To identify genes that may be regulated by IdeR, we searched the *M.tuberculosis* genome for iron boxes that were in untranslated regions within 200 bp of an open reading frame. We chose two half-site sequences with allowance for a variable number of intervening base pairs for our search. In the 4.41MB of the *M.tuberculosis* genome (24), 58 sequences with acceptable homology to the consensus sequence were identified. Six of these were in untranslated regions and had corresponding downstream open reading frames.

A DNA gel binding assay was used to assess the ability of DtxR to bind to these putative iron-regulated operator regions drawn from the *M.tuberculosis* genome. Figure 8 shows the results of gel binding assays using ^{32}P-end-labeled 100 bp DNA fragments containing five of the putative iron boxes (IB1-5). Binding of DtxR to the *tox* operator could be abolished with the addition of unlabeled *tox* DNA, but not with nonspecific DNA. All five of these putative iron boxes were bound by DtxR to a similar degree as that observed with the *tox* operator. The iron box upstream of the *narG* homologue, IB6, did not bind to DtxR. (data not shown)

Table 3 identifies the open reading frames (ORF) downstream of these six iron boxes. BLAST searches reveal that these genes encode a PhoP homologue (a transmembrane sensor of a two-component sensor-regulator pair), a homologue of the HtrA serine protease, 16S ribosomal RNA, an alcohol dehydrogenase AdhB, and a homologue of the *M.tuberculosis* 19kDa antigen (a protein shown to be involved in the human immune response to tuberculosis). (25) IB6, which was not shifted by DtxR *in vitro*, appears upstream of a nitrate reductase subunit gene, *narG*.

Discussion

The concentration of free ferrous iron (Fe^{2+}) is extremely limited *in vivo*. For this reason, many pathogenic prokaryotes such as *Vibrio cholerae, E.coli, Neisseria gonorrheae,* and *Corynebacterium diphtheriae* co-regulate virulence gene expression with iron sensing and scavenging systems. (26-28) In *C.diphtheriae,* one such mechanism of iron regulation relies on a repressor, DtxR, which binds to a specific palindromic sequence in the operator regions of the genes that it controls. (29) In low iron states, the metal-ion triggered conformational change that allows it to bind to the DNA is disrupted, the repressor loses affinity for the operator site, and gene expression occurs. Recently, a positive dominant DtxR(E175K) mutant unresponsive to iron was generated by random PCR mutagenesis using a genetic selection system. (10)

Significant amino acid identity between corynebacterial DtxR and mycobacterial IdeR has been described. In the amino terminal 140 amino acids there is a DNA binding helix-turn-helix motif, a primary metal ion binding site and a protein-protein interaction domain. Corynebacterial DtxR and mycobacterial IdeR share 80% amino acid identity in this portion of both proteins. Evidence of functional homology between IdeR and DtxR has been shown previously by Schmitt *et al.* (8)

The results show that the positive dominant DtxR(E175K) iron-independent repressor is expressed in the phylogenetically related mycobacteria. Furthermore, we have shown that it is dominant and constitutively attenuates *M.tuberculosis* in a murine model of infection. Rational attenuation of *M.tuberculosis* provides the opportunity to define specific virulence factors of the organism and the development of live vaccines superior to BCG. However, gene replacement has proven difficult in *M.tuberulcosis* due to high rates of illegitimate recombination. Addition of a dominant mutant gene is technically simpler than gene replacement in *M.tuberculosis* and permits comparison of a defined merodiploid strain with an isogenic wild type strain. There are reports of *E.coli* genes introduced into other bacteria to regulate the expression of endogenous genes. In a paper by de Henestrosa *et al.*, a mutant *E.coli recA* gene produced aberrancies in SOS gene

induction when expressed in heterologous gram-negative systems. (30) Although a *M.tuberculosis* strain containing empty plasmid was not compared with wild type *M.tuberculosis*, the *in vitro* Bactec comparison showed no differences in the rate of growth of the mutant strain as compared to wild type. In addition, studies of deletion mutants have shown that plasmid complementation fully restores virulence suggesting that there is little cost to the organism to maintain the plasmid. (31, 32) This strain is the first example of the use of a dominant positive gene from another pathogenic prokaryote to attenuate the virulence of *M.tuberculosis*.

Animal models have shown that inactivation and clearance of virulent *M.tuberculosis* in liver and spleen is effectively accomplished, but that the same cell-mediated immune mechanisms appear relatively ineffective in lungs. These data point to a difference in the intracellular microenvironment of the lung granuloma. (33) It has been postulated from BCG and H37Ra data that avirulent or attenuated strains lack the genes required for effective growth within lung phagocytes. (34) The data suggest that IdeR may regulate genes important for *M.tuberculosis* survival late in lung infection as attenuation seems to increase dramatically at 12 weeks after infection. This may correlate with the onset of granuloma formation in mouse lungs and the need for *M.tuberculosis* to scavenge iron from extracellular rather than intracellular sources. (35)

The *ideR* gene has been found in *M.tuberculosis, M.bovis,* and *M.smegmatis.* In *M.smegmatis,* an *ideR* mutant showed defective regulation of siderophore biosynthesis. (9) Potential IdeR binding sites upstream of exochelin biosynthesis genes such as *fxbA* have recently been confirmed. (36, 37) In addition, several IdeR recognition sequences have been identified using computer searches of the *M.tuberculosis* genome. (38) We have similarly identified 6 potential IdeR-binding sites in *M.tuberculosis,* 5 of which demonstrated significant binding with DtxR in a gel shift assay. We postulate that the sixth sequence was unable to bind in our *in vitro* assay because of incorrect spacing between the two relatively well-conserved half-sites. We used DtxR rather than IdeR in this gel-shift assay because we specifically sought to identify genes responsible for the attenuated phenotype of *M.tuberculosis* DtxR(E175K). The predicted ORF downstream of IB-1 encodes a

homologue of *phoP*, a phosphotransfer response regulator. A number of two-component pairs have been shown to regulate virulence pathways in bacterial pathogens. These include BvgA/BvgS in *Bordatella pertussis,* VanR/VanS in *Enterococcus faecium,* PhoP-PhoQ in *Salmonella typhimurium* , and OmpT/EnvZ in *Shigella flexneri.* (39, 40) In *M.tuberculosis,* a two-component pair, *mtrA-mtrB*, has been previously described and appears to play an intracellular role as expression of *mtrA* increases upon entry into macrophages. (41) Furthermore, *phoP*- mutants in *Salmonella* are unable to synthesize many of the proteins expressed on interaction with macrophages. (42) Downstream of IB-2 is *adhB*, an alcohol dehydrogenase. In*Salmonella typhimurium*, it has been postulated that alcohol dehydrogenase genes such as *eutG* may confer a protective role from reactive aldehyde intermediates associated with inflammatory cell activation. (43)

IB-3 lies upstream of an ORF homologous to a HtrA-like serine protease which, in *E.coli* are thought to be required for growth of the organism at high temperature, and may play a role in degrading abnormal proteins within the periplasm. (44, 45) It is a known virulence factor in several organisms including *Salmonella typhimurium, Yersinia enterocolitica, Brucella abortus,* and *Brucella melitensis.* (46-49) In an animal protection model, a *Salmonella typhimurium htrA* mutant is attenuated and a safe and immunogenic live vaccine strain in mice. (50) Both *Mycobacterium avium* subsp. *paratuberculosis* and *M.tuberculosis* have putative serine proteases with significant homology to HtrA. (24, 51)

IB-4 lies upstream of *rrnA,* a 16S rRNA gene which has been shown to be part of a group of rDNA operons in both slow and fast-growing mycobacteria with hypervariable multiple promoter regions (HMPR). The *M.tuberculosis rrnA* operon has 2 promoters one of which is conditionally induced suggesting complex regulation of this esential gene. (52)

Our results indicate that a dominant positive corynebacterial *dtxR* allele attenuates the virulence of *M.tuberculosis* in a murine model. These data implicate the *M.tuberculosis* IdeR repressor as a regulator of genes essential for full virulence.

Table 2:

Strain/Plasmid	Genotype/Description	Source/Reference
Plasmids		
PNBV1	*E.coli*-mycobacterial shuttle vector Ap^R, Hy^R	(12)
pSDM2	pSC101-derivative containing the *dtxR*(E175K) gene, Km^R	(10)
pNBV1/SAD	pNBV1 containing the *dtxR*(E175K) gene, Ap^R, Hy^R	this paper
Strains		
E.coli DH5	F^- *recA1, hsdR17, thi-1, gyrA96, supE44, endA1, relA1, recA1, deoR, (lacZYA-argF)* U169 (80 *lacZ* M15)	(14)
M.smegmatis mc^26 1-2C	transformable variant of mc^26	(13)
M.tuberculosis CDC1551	virulent recent clinical isolate	(11)
M.tuberculosis DtxR(E175K)	pNBV1/SAD	this paper

Hy hygromycin, Ap ampicillin, Km kanamycin

Table 3: Iron Boxes (IB)

Name	Downstream ORF	Accession Number	Description
IB-1	PhoP homologue	Rv0761c	Two-component phosphotransferase

			regulatory protein
IB-2	AdhB	Rv0757	Alcohol dehydrogenase
IB-3	HtrA homologue	Rv0983	Serine protease, HtrA-antigen family
IB-4	RrnA	MTB00368	16S ribosomal RNA protein
IB-5	Hypothetical protein	Rv3764c	Predicted ORF with 26% similarity to *M.tuberculosis* 19kDa antigen beginning at base 4,210,314

Example 2

Attenuation of Staphylococcus Infection in a Murine Model

In Example 1 Applicants demonstrated the ability of *in vitro* constructed and tested metal ion independent mutant gene of a normally metal ion dependent transcriptional repressor to attenuate virulence in a pathogenic bacterium of a different species. The metal ion dependent repressor was from *C. diphtheriae* and the pathogenic species was *M. tuberculosis*. The sequence homology of DtxR and the endogenous mycobacterial DtxR –like repressor IdeR is approximately 60% [approaching 90% in the N-terminal half of the repressors]. In example two, applicants have expanded this observation to demonstrate that a dominant metal ion independent repressor such a the mutant E175K DtxR is capable of attenuating virulence across wider species differences in a bacterial strain which co-regulates virulence with iron concentrations and in which there is a Fur or Fur-like repressor. (Heidrich et al (1996) FEMS 140 253-259, Trivier and Courcol (1996) FEMS 141 117-127).

Briefly and a described above in Example 1, partial diploid analysis in reporter host strains of *Escherichia coli* was performed revealing DtxR(E175K) is dominant in strains which carry DtxR/DtxR(E175K). A recombinant partial diploid [*dtx*R(E175K) / *Sir*R] of *Staphylococcus aureus* was constructed carrying the iron independent E175K mutant DtxR. When this strain was used in a series of mouse challenge experiments, it was found to attenuate virulence in a mouse model of staphylococcal infection [See Figures 9A and 9B] When this strain is compared to the parent strain in a mouse skin lesion model there was a significant decrease in CFU isolated from the lesion after 8 days associated with a significant decrease in lesion size compared to *wt* at each time point through out the study [See Figures 9A

and 9B]. These data further demonstrate that activated E175K DtxR or E175K DtxR/DtxR–like (IdeR or SirR) heterodimeric repressors decrease the virulence of pathogenic microorganisms.

It is noteworthy that a dominant metal ion independent repressor from *C. diphtheriae* suppresses virulence in *S. aureus,* especially since the homology of the two repressors is at the amino acid level approaches only 30% as compared to the nearly 60% identity between IdeR from *M. tuberculosis* and DtxR. This means that dominant metal ion independent repressors can be employed across species barriers to selectively control gene expression to produce desired phenotypical changes.

References

1. Litwin, C. M. & Calderwood, S. B. (1993) *Clin. Microbiol. Rev.* **6,** 137-149.

2. Kochan, I. (1973) *Curr. Top. Microbiol. Immunol.* **60,** 1-30.

3. Raghu, B., Raghupati, S. & Venkatesan, P. (1993) *Biochem. Mol. Biol. Int.* **31,** 341-3 48.

4. Murray, M. J., Murray, A. B., Murray, M. B. & Murray, C. J. (1978) *Br. Med. J.* **2,** 1113-1115.

5. Gordeuk, V. R., McLaren, C. E., MacPhail, A. P., Deichsel, G. & Bothwell, T. H. (1996) *Blood* **87,** 3470-3476.

6. White, A., Ding, X., vanderSpek, J., Murphy, J. R. & Ringe, D. (1998) *Nature* **394,** 502-6.

7. Doukhan, L., Predich, M., Nair, G., Dussurget, O. & Mandic-Mulec, I. (1995) *Gene* **165,** 67-70.

8. Schmitt, M. P., Predich, M., Doukhan, L., Smith, I. & Holmes, R. K. (1995) *Infect. Immun.* **63,** 4284-4289.

9. Dussurget, O., Rodriguez, M. & Smith, I. (1996) *Mol. Microbiol.* **22,** 535-544.

10. Sun, L., vanderSpek, J. & Murphy, J. R. (1998) *Proc. Natl. Acad. Sci. USA* .**95**, 14985-14990

11. Valway, S. E., Sanchez, M. P. C., Shinnick, T. F., Orme, I., Agerton, T., Hoy, D., Jones, J. S., Westmoreland, H. & Onorato, I. M. (1998)*N. Engl. J. Med.* **338,** 633-639.

12. Howard, N. S., Gomez, J. E., Ko, C. & Bishai, W. R. (1995) *Gene* **166,** 181-182.

13. Zhang, Y., Lathigra, R., Garbe, T., Catty, D. & Young, D. (1991) *Mol. Microbiol.* **5,** 381-391.

14. Laboratories, B. R. (1986) in *Bethesda Research Laboratory Focus*, Vol. 8, pp. 9.

15. Jacobs, W. R., Jr., Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G. & B.R., B. (1991) *Methods Enzymol.* **204,** 537-555.

16. Ausubel, F. M., R., B., R.E., K., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994) *Current Protocols in Molecular Biology* (Wiley, New York).

17. Tao, X., Boyd, J. & Murphy, J. R. (1992) *Proc. Natl. Acad. Sci. USA* .

18. Miyazaki, E., Miyazaki, M., Chen, J. M., Chaisson, R. E. & Bishai, W. R. (1999) *Antimicrob. Agents Chemother.* **43,** 85-89.

19. Hendrickson, W. & Schleif, R. F. (1984) *J. Mol. Biol.* **178,** 611-628.

20. Tao, X., Zeng, H.-y. & Murphy, J. R. (1995)*Proc. Natl. Acad. Sci. USA* **92,** 6803-6807.

21. Saviola, B., Seabold, R. & Schleif, R. F. (1998) *J. Mol. Biol.* **278,** 539-548.

22. Tao, X., Schiering, N., Zeng, H.-y., Ringe, D. & Murphy, J. R. (1994) *Mol. Microbiol.* **14,** 191-197.

23. Schmitt, M. P. & Holmes, R. K. (1994) *J. Bacteriol.* **176,** 1141-1149.

24. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., III, C. E. B., Tekaia, F., Badcock,

K., Basham, D., Brown, D., Chillingworth, T., R., C., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Kroghs, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M.-A., Roger, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S. & Barell, B. G. (1998)*Nature* **393,** 537-544.

25. Husson, R. N. & Young, R. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1679-1683.

26. Goldberg, M. B., DiRita, V. J. & Calderwood, S. B. (1990) *Infect. Immun.* **58,** 55-60.

27. Calderwood, S. B. & Mekalanos, J. J. (1987)*J. Bacteriol.* **169,** 4759-4764.

28. Morse, S. A., Chen, C., LeFaou, A. & Mietzner, T. A. (1988) *Rev. Infect. Dis.* **10,** s306-s310.

29. Boyd, J., Oza, M. N. & Murphy, J.R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5968-5972.

30. de Henestroza, A. R. F., Calero, S. & Barbe, J. (1991)*Mol. Gen. Genet.* **226,** 503-506.

31. Berthet, F.-X., Lagranderie, M., Gounon, P., Laurent-Winter, C., Ensergueix, D., Chavarot, P., Thouron, F., Maranghi, E., Pelicic, V., Portnoi, D., Marchal, G. & Gicquel, B. (1998) *Science* **282,** 759-762.

32. Jackson, M., Phalen, S. W., Lagranderie, M., Ensergueix, D., Chavarot, P., Marchal, G., McMurray, D. N., Gicquel, B. & Guilhot, C. (1999) *Infect. Immun.* **67,** 2867-2873.

33. Collins, F. M. (1993) *Crit. Rev. Microbiol.* **19,** 1-16.

34. Pascopella, L., Collins, F. M., Margin, J. M., Jacobs, W. R. J. & Bloom, B. R. (1993) *Infect. Agents dis.* **2,** 282-284.

35. Long, E. R. (1958) in *The chemistry and chemotherapy of tuberculosis*, ed. Long, E. R. (Williams & Wilkins, Baltimore), pp. 163-182.

36. Fiss, E. H., Yu, S. & Jacobs, W. R. J. (1994)*Mol. Microbiol.* **14,** 557-569.

37. Dussurget, O., Timm, J., Gomez, M., Gold, B., Yu, S., Sabol, S. Z., Holmes, R. K., Jacobs, W. R. & Smith, I. (1999)*J. Bacteriol.* **181,** 3402-3408.

38. Dussurget, O. & Smith, I. (1998) *Trends Microbiol.* **6,** 354-8.

39. Groisman, E. A. & F., H. (1995) in *Two-component signal transduction*, eds. Hoch, J. A. & Silhavy, T. J. (ASM Press, Washington, D.C.), pp. p319-332.

40. Dziejman, M. & Mekalanos, J. J. (1995) in *Two-Component Signal Transduction*, eds. Hoch, J. A. & Silhavy, T. J. (ASM Press, Washington, D.C.), pp. 305-317.

41. Via, L. E., Curcic, R., Mudd, M. H., Dhandayuthapani, S., Ulmer, R. J. & Deretic, V. (1996)*J. Bacteriol.* **178,** 3314-3321.

42. Buchmeier, N. A. & Heffron, F. (1990) *Science* **248,** 730-732.

43. Stojiljkovic, I., Baumler, A. J. & Heffron, F. (1995) *J. Bacteriol.* **177,** 1357-1366.

44. Lipinska, B., Fayet, O., Baird, L. & Georgopoulos, C. (1989) *J. Bacteriol.* **171,** 1574-1584.

45. Strauch, K. L. & Beckwith, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1576-1580.

46. Johnson, K., Charles, I., Dougan, G., Pickard, K., O'Gaora, P., Costa, G., Ali, T., Miller, I. & Hormaeche, C. (1991) *Mol. Microbiol.***5,** 401-407.

47. Yamamoto, T., Hanawa, T., Ogata, S. & Kamiya, S. (1996) *Infect. Immun.* **64,** 2980-2987.

48. Robertson, G. T., Elzer, P. H. & II, R. M. R. (1996) *Vet. Microbiol.* **49,** 197-207.

49. Phillips, R. W., Elzer, P. H. & II, R. M. R. (1995)*Microb. Pathog.* **19,** 227-284.

50. Chatfield, S., Strahan, K., Pickard, D., I., C., Hormaeche, C. E. & Dougan, G. (1992) *Microb. Pathog.* **12,** 145-151.

51. Cameron, R. M., Stevenson, K., Inglis, N. F., Klausen, J. & Sharp, J. M. (1994) *Microbiol.* **140,** 1977-1982.

52. Gonzalez-Y-Merchand, J. A., Garcia, M. J., Gonzalez-Rico, S., Colston, M. J. & Cox, R. A. (1997)*J. Bacteriol.* **179,** 6949-6958.

All publications cited in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein incorporated by reference to the same extent as if each individual publication were specifically and individually indicated to be incorporated by reference. In addition, U.S. Provisional Application Nos. 60/161,193, filed October 22, 1999, and 60/161,292, filed October 25, 1999, are hereby incorporated by reference.

Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the appended claims.

INDUSTRIAL APPLICABILITY

The present invention is applicable to human and veterinary medicine and more specifically to disease prevention and control of infectious and disease.

5

CLAIMS

1. A composition comprising a virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage, said prokaryote containing a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a dominant, metal ion-independent repressor protein or a partially metal ion independent repressor protein, and a carrier.

2. The composition of claim 1 wherein said recombinant DNA molecule is contained in a non-chromosomal vector.

3. The composition of claim 1 wherein said prokaryote is a bacterium.

4. The composition of claim 3 wherein said bacterium is a member of the genus *Mycobacterium*.

5. The composition of claim 4 wherein said bacterium is *Mycobacterium tuberculosis*.

6. The composition of claim 4 wherein said bacterium is *Mycobacterium leprae*.

7. The composition of claim 4 wherein said bacterium is *Mycobacterium avium*.

8. The composition of claim 4 wherein said bacterium is *Mycobacterium paratuberculosis*.

9. The composition of claim 4 wherein said bacterium is *Mycobacterium bovis*.

10. The composition of claim 3 wherein said bacterium is a member of the genus *Staphylococcus*.

11. The composition of claim 10 wherein said bacterium is *Staphylococcus epidermitis*.

12. The composition of claim 10 wherein said bacterium is *Staphylococcus aureus*.

13. The composition of claim 3 wherein said bacterium is a member of the genus *Streptococcus*.

14. The composition of claim 13 wherein said bacterium is *Streptococcus mutans*.

15. The composition of claim 13 wherein said bacterium is *Streptococcus pneumoniae.*

16. The composition of claim 1 wherein said sequence encodes a metal ion-independent diphtheria toxin repressor (DtxR) protein.

17. The composition of claim 1 wherein said sequence encodes a metal ion-independent or a partially metal ion independent IdeR or SirR repressor protein.

18. The composition of claim 3 wherein said bacterium is a gram-positive bacterium.

19. A composition comprising a virulent or opportunistic bacterium in which metal ion-dependent gene regulation confers a growth or an infectious advantage, said bacterium containing a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a metal or metal ion-independent DtxR protein or a partially metal ion independent DtxR protein, and a carrier.

20. An isolated and purified DNA molecule consisting essentially of a sequence encoding a metal ion independent or a partially metal ion independent DtxR or homologue thereof.

21. The DNA molecule of claim 20 wherein said DtxR homolog is IdeR.

22. The DNA molecule of claim 20 wherein said DtxR homolog is SirR.

23. A recombinant DNA molecule containing a constitutive promoter element in operable association with the DNA molecule of claim 20.

24. A recombinant vector comprising a promoter element in operable association with the DNA molecule of claim 20.

25. The recombinant vector of claim 24 which is a plasmid.

26. A virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage, wherein said prokaryote is transformed with a DNA molecule encoding a dominant, metal ion-independent repressor protein or a partially metal ion-independent repressor protein, and wherein said DNA molecule is expressed in said prokaryote.

27. A method of enhancing protective immunity against infection or disease caused by an opportunistic or virulent prokaryotic pathogen in which metal ion-dependent gene regulation confers a growth or an infectious advantage, comprising administering to an animal the composition of claim 1.

28. The method of claim 27 wherein said animal is a human.

29. The method of claim 27 wherein the prokaryote is in live form.

30. The method of claim 27 wherein the prokaryote is in inactivated form.

31. A method of attenuating or reducing the severity of an infection or disease caused by an opportunistic or virulent prokaryotic pathogen in which metal ion-dependent gene regulation confers a growth or an infectious advantage, comprising administering to an animal the composition of claim 1.

32. The method of claim 1 wherein said animal is a human.

```
                   1              15 16            30 31            45 46            60 61            75 76            90
1 mtbider          MNELVDTTEMYLRTI YDLEEEGVTPLRARI AERLDQSGPTVSQTV SRMERDGLLRVAGDR HLELTEKGRALAIAV MRKHRLAERLLVDVI      90
2 dtxr             MKDLVDTTEMYLRTI YELEEEGVTPLRARI AERLEQSGPTVSQTV ARMERDGLVVVASD- SLQMTPTGRTLATAV MRKHRLAERLLTDII      89
3 staphepisirr     --------------- ---MTVSCPPPS--T SEREEQ-----ARAL CLRLLTARSRTRAEL AGQLAKRGYPEDIGN RVLDRLAAVGLVDDT      65

                   91            105 106           120 121           135 136           150 151           165 166           180
1 mtbider          GLPWEEVHAEACRWE HVMSEDVERRLVKVL NNPTTSPFGNPIPGL VELGVGPEPGADDAN LVRLTELPAGSPVAV VVRQLTEHVQGDIDL      180
2 dtxr             GLDINKVHDEACRWE HVMSDEVERRLVKVK DVSR-SPFGNPIPGL DELGVGNS--DAAAP GTRVIDAATSMPRKV RIVQINEIFQVETDQ      176
3 staphepisirr     DFAEQWVQSRRANAA KSKRALAAELHAKGV DDDVITTVLGGIDAG AERGRAEK--LVRAR LRREVLIDDGTDEAR VSRRLVAMLARRG-Y      152

                   181           195 196           210 211           225 226           240 241           255 256           270
1 mtbider          ITRLKDAGVVPNARV TVETTPGGGVTIVIP GHENVTLPHEMAHAV KVEKV      230
2 dtxr             FQLLDADIRVGSEVE IVDRD--GHITLSHN GKD-VELLDDLAHTI RIEEL      223
3 staphepisirr     GQTLACEVVIAELAA ERERR--RV------ --------------- -----      174
```

FIGURE 1

```
         1              15 16          30 31          45 46          60 61          75 76          90
1 Ml.    MNDLVDTTEMYLRTI YDLEEEGIVTPLRAR IAER-----PTVSQT VSRMERDGLLRVAGN RHLELTTKGRAMAIA VMRKHRLAERLLVDV    85
2 TB     MNELVDTTEMYLRTI YDLEEEG-VTPLRAR IAERLDQSGPTVSQT VSRMERDGLLRVAGD RHLELTEKGRALAIA VMRKHRLAERLLVDV    89
3 Ms     MNDLVDTTEMYLRTI YDLEEEG-VVPLRAR IAERLDQSGPTVSQT VSRMERDGLLHVAGD RHLELTDKGRALAVA VMRKHRLAERLLVDV    89
4 Mtb    MTVSCPPPSTSER-- ---EEQARALCLRLL TARS-----RTRAEL AGQLAKRGYPEDIGN RVLD-----RLAAVG LVDDTDFAEQ-----    70

         91             105 106        120 121        135 136        150 151        165 166        180
1 Ml.    IGLPWEEVHAEACRW EHVMSEDVERRLIKV LNNPTTSPFGNPIPG LLDLGAGPDASAANA KLVRLTELPSGSPVA VVVRQLTEHVD-DID   174
2 TB     IGLPWEEVHAEACRW EHVMSEDVERRLVKV LNNPTTSPFGNPIPG LVELGVGPEPGADDA NLVRLTELPAGSPVA VVVRQLTEHVQGDID   179
3 Ms     IGLPWEDVHAEACRW EHVMSEEVERRLVQV LENPTTSPFGNPIPG LTELAVTPGVNTEDV SLVRLTELPVGMPVA VVVRQLTEHVQGDTD   179
4 Mtb    ----WVQS-RRANAA KSKRALAAELHAKGV DDDVITTVLGG-IDA GAERGR--AEKLVRA RLRREVLIDDGTDEA RVSRRLVAMLA----   148

         181            195 196        210 211        225 226        240 241        255 256        270
1 Ml.    LITRLKDTGVVPNAR VTVETSPAGNVIIII PGHENVTLPHEMAHA VKVEKV---         225
2 TB     LITRLKDAGVVPNAR VTVETTPGGGVTIVI PGHENVTLPHEMAHA VKVEKV---         230
3 Ms     LIGRLKEAGVVPNAR VTVEANNNGGVMIVI PGHEQVELPHHMAHA VKVEKVEKV         233
4 Mtb    ---RRGYGQTLACEV VIAELAAERERRRV- --------------- ---------         174
```

FIGURE 2

```
1       10          20          30          40          50          60
Bl DtxR       MKDLVDTTEM YLRTIYELEE  EGIVPLRARI  AERLEQSGPT VSQTVARMER DGLVHVSPDR
Cd DtxR       MKDLVDTTEM YLRTIYELEE  EGVTPLRARI  AERLEQSGPT VSQTVARMER DGLVVVASDR
Mt IdeR       MNELVDTTEM YLRTIYDLEE  EGVTPLRARI  AERLDQSGPT VSQTVSRMER DGLLRVAGDR
Ms IdeR       MNDLVDTTEM YLRTIYDLEE  EGVVPLRARI  AERLDQSGPT VSQTVSRMER DGLLHVAGDR
Ml IdeR       MNDLVDTTEM YLRTIYDLEE  EGIVTPLRARI AER      PT VSQTVSRMER DGLLRVAGNR
Sl DesR       MSGLIDTTEM YLRTILELEE  EGVVPMRARI  AERLDQSGPT VSQTVARMER DGLVSVAADR
Se SirR      M  LTEEKED YLKAILTNDG  DVSFVSNKKL  SQFLNIKPPS VSEMVGRLEK EGYVETKHYK
Sa SirR       M  LTEEKED YLKAILTNNG  DKNFVTNKIL  SQFLNIKPPS VSEMVGRLEK AGYVETKPYK
Ef            M   TPNRED YLKLIFELGG  DEVKVNNKQI  VSGLDVSAAS VSEMISKLVK EDLVEHSPYQ
Sg            M   TPNKED YLKCLYELGT  RHNKITNKEI  AGLMQVSPPA VTEMMKKLLA EELLIKDKKA
Sm            M   TPNKED YLKIIYELSE  RDEKISNKQI  AEKMSVSAPA VSEMVKKLLL EDLVLKDKQA
Spn           M   TPNKED YLKCIYEIGI  DLHKITNKEI  AARMQVSPPA VTEMIKRMKS ENLILKDKEC
Spy           M   TPNKED YLKCIYEIGI  DLHKITNKEI  AARMQVSPPA VTEMIKRMKS ENLILKDKEC

CONSENSUS     M--*----E* YL*-I*----  ------NK-*  *-------P- ****--**-* *-L-------

                    70          80          90          100         110         120
Bl DtxR       SLEMTPEGRS LAIAVMRNDR LAERLLTDII GLDIHKVHDE ACRWEHVMSD EVERRLVEVL
Cd DtxR       SLQMTPTGRT LATAVMRKHR LAERLLTDII GLDINKVHDE ACRWEHVMSD EVERRLVKVL
Mt IdeR       HLELTEKGRA LAIAVMRKHR LAERLLVDVI GLPWEEVHAE ACRWEHVMSE DVERRLVKVL
Ms IdeR       HLELTDKGRA LAVAVMRKHR LAERLLVDVI LPWEDGVHAE ACRWEHVMSE EVERRLVQVL
Ml IdeR       HLELTTKGRA MAIAVMRKHR LAERLLVDVI GLPWEEVHAE ACRWEHVMSE DVERRLIKVL
Sl DesR       HLELTDEGRR LATRVMRKHR LAECLLVDVI GLEWEQVHAE ACRWEHVMSE AVERRVLELL
Se SirR      GARLTEEGLK QTLDIIKRHR LLRLFLIEIL QYNWEEVHQE AEILEHRISD LFVERLDKIL
Sa SirR       GVRLTEDGLT HTLDII RHR LLELFLIEIL KYNWEEVHQE AEILEHRISD LFVERLDSLL
Ef            GVQLTEKGLK KASTLIRKHR IWEVFLVEHL NYTWNDVHEE AEVLEHVTSQ TLVNRLADYL
Sg            GYLLTDLGLK LVSDLYRKHR LIEVFLVHHL GYTTEEIHEE AEVLEHTVSD HFVERLDQLL
Sm            GYLLTKKGQI LASSLYRKHR LIEVFLMNHL NYTADEIHEE AEVLEHTVSD VFVERLDKFL
Spn           GYLLTDLGLK LVSELYRKHR LIEVFLVHHL DYTSDQIHEE AEVLEHTVSD LFVERLDKLL
Spy           GYLLTDLGLK LVSELYRKHR LIEVFLVHHL DYTSDQIHEE AEVLEHTVSD LFVERLDKLL

CONSENSUS     ---LT-G-- -------**HR L-*-*L---L -------H-E A*-*EH-S* -***R***-L

              130         140         150         160         170         180
Bl DtxR       DDVHRSPFGN PIPGLGEIGL DQADEPDSGV RAIDLPLGEN LKARIVQLNE ILQVDLEQFQ
Cd DtxR       KDVSRSPFGN PIPGLDELGV GNSDAAAPGT RVIDAATSMP RKVRIVQINE IFQVETDQFT
Mt IdeR       NNPTTSPFGN PIPGLVELGV GPEPGADDAN LVRLTELPAG SPVAVVVRQL TEHVQGDIDL
Ms IdeR       ENPTTSPFGN PIPGLTELAV TPGVNTEDVS LVRLTELPVG MPVAVVVRQL TEHVQGDTDL
Ml IdeR      NNPTTSPFGN PIPGLLDLGA GPDASAANAK LVRLTELPSG SPVAVVVRQL TEHV DDIDL
Sl DesR       RHPTESPYGN PIPGLEELGE TDGADPFLDE GMVSLADLDP GQEGKTVVVR RIGEPIQTDA
Se SirR      NFPKTCPHGG VIP       R GNSDAAAPGT SILNFEPGER     VTVRRV RRDK  TELL
Sa SirR       NFPETCPHGG VIP       R NNEYKEKYIT TILNYEPGDI     VTIKRV RDK   TDLL
Ef            NHPEFCPHGG VIPEDNQPIH EEKRQTLTDY PVGTKIRIAR VLDEKELLDY LVSIDLNIQE
Sg            DYPKACPHGG TIPAKGELLV EKHKLTLEEA KEKGDYILAR VHDNFDLLTY LERNGLQVGK
Sm            NYPKVCPHGG TIPGHGQPLV ERYRTTLKGV TEMGVYLLKR VQDNFQLLKY MEQHHLKIGD
Spn           GFPKTCPHGG TIPAKGELLV EINNLPLADI KEAGAYRLTR VHDSFDILHY LDKHSLHIGD
Spy           GFPKTCPHGG TIPAKGELLV EINNLPLADI KEAGAYRLTR VHDSFDILHY LDKHSLHIGD

CONSENSUS     --P--*P*G* -IP---**-- ---------- ---------  ---------- ----------
```

FIGURE 3

```
                    190         200         210         220         230
Bl DtxR       ALTDAGVEIG TEVDIINEQG RVVITHNGSS VELIDDLAHA VRVEKVEG
Cd DtxR       QLLDADIRVG SEVEIVDRDG HITLSHNGKD VELLDDLAHT IRIEEL
Mt IdeR       ITRLKDAGVV PNARVTVETT PGGGVTIVIP GHENVTLPHE MAHAVKVEKV
Ms IdeR       IGRLKEAGVV PNARVTVEAN NNGGVMIVIP GHEQVELPHH MAHAVKKKVE KVEKV
Ml IdeR       ITRLKDTGVV PNARVTVETS PAGNVIIIIP GHENVTLPHE MAHAVKVEKV

Sl DesR        QLMYTLRRAG VQPGSVVSVT ESAGGVLVGS GGEAAELEAD TASHVFVAKR
Sa SirR       VYLSSKDIYI GNTVEIVSKD DTNKVIILKR NDIVTILSYE NAMNIFAEK
Sa SirR        IYLSSKDISI GNEVEIVSKD EMNKVIIIKR NDNVIIVSYE NAMNMFAEK
Ef             EYTIKEIAAY EGPITIYNEN KELSVSFKAA NTIFVEPLIR ESEEN
Sg SgoR        TIRFLGYDDF SHLYSLEVDG QEIQLAQPIA QQIYVEKI
Sm SloR        ELRLLEYDAF AGAYTIEKDG EQLQVTSAVA SQIYIEKKAY
Spn            QLQVKQFDGF SNTFTILSND EDLQVNMDIA KQLYVEKIN
Spy            QLQVKQFDGF SNTFTILSND EDLQVNMDIA KQLYVEKIN

CONSENSUS     ---------- ---------- ---------- ---------- ----------
```

FIGURE 3 cont.

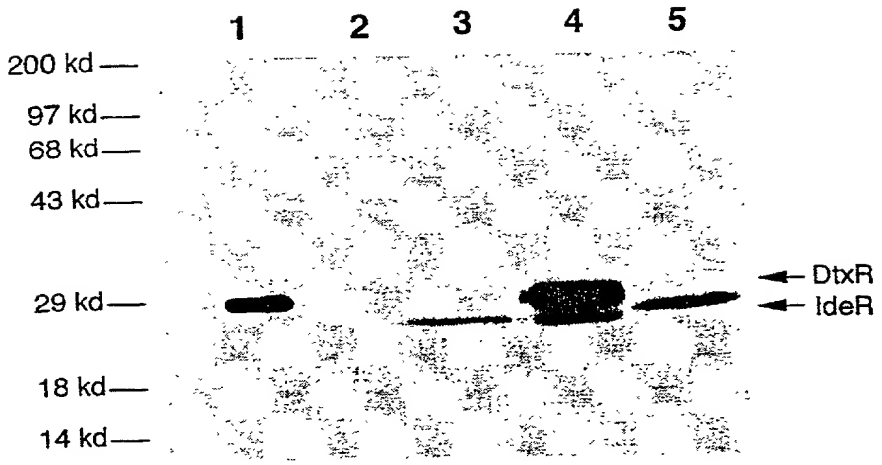


FIGURE 4

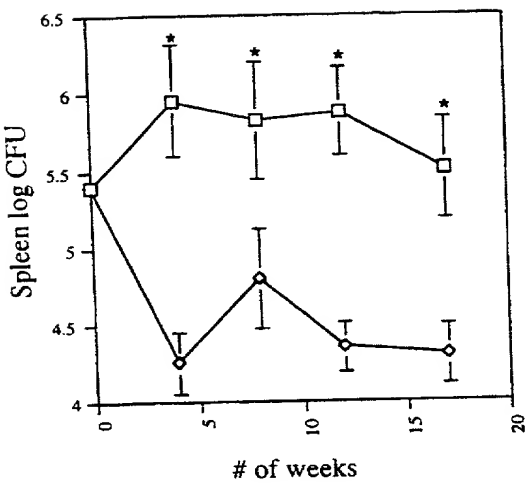


FIGURE 5A

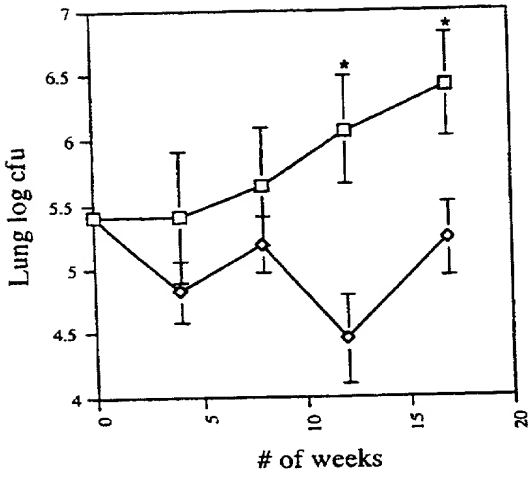
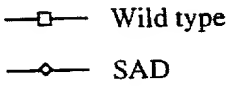


FIGURE 5B



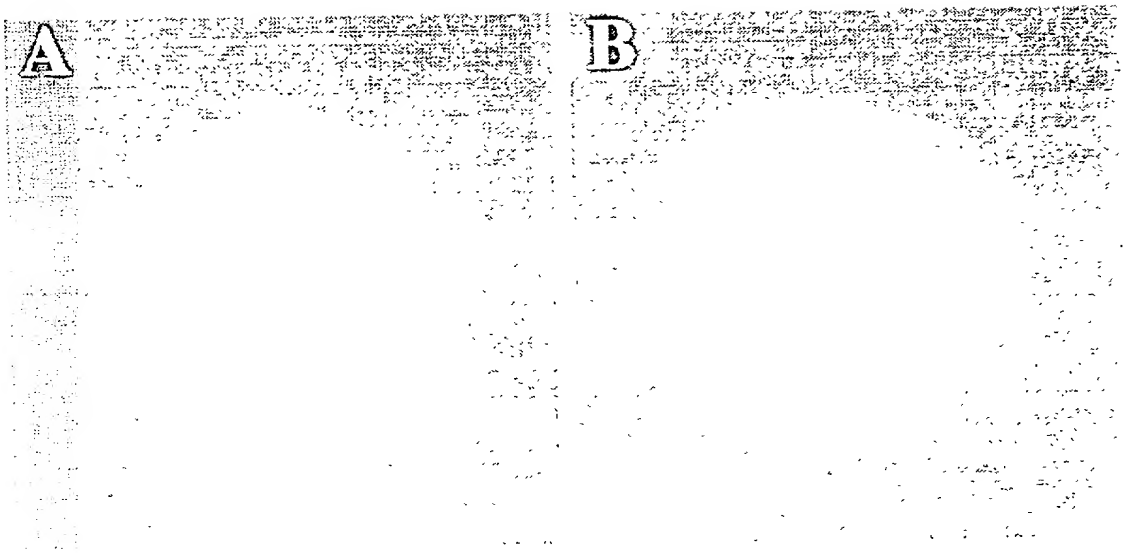


FIGURE 6A

FIGURE 6B

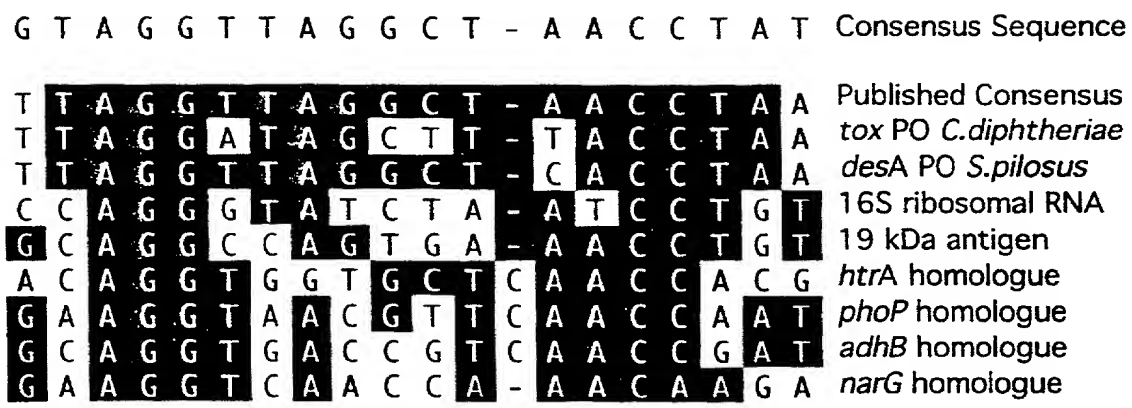

G T A G G T T A G G C T - A A C C T A T Consensus Sequence
T T A G G T T A G G C T - A A C C T A A Published Consensus
T T A G G A T A G C T T - T A C C T A A tox PO C.diphtheriae
T T A G G T T A G G C T - C A C C T A A desA PO S.pilosus
C C A G G G T A T C T A - A T C C T G T 16S ribosomal RNA
G C A G G C C A G T G A - A A C C T G T 19 kDa antigen
A C A G G T G G T G C T C A A C C A C G htrA homologue
G A A G G T A A C G T T C A A C C A A T phoP homologue
G C A G G T G A C C G T C A A C C G A T adhB homologue
G A A G G T C A A C C A - A A C A A G A narG homologue

FIGURE 7

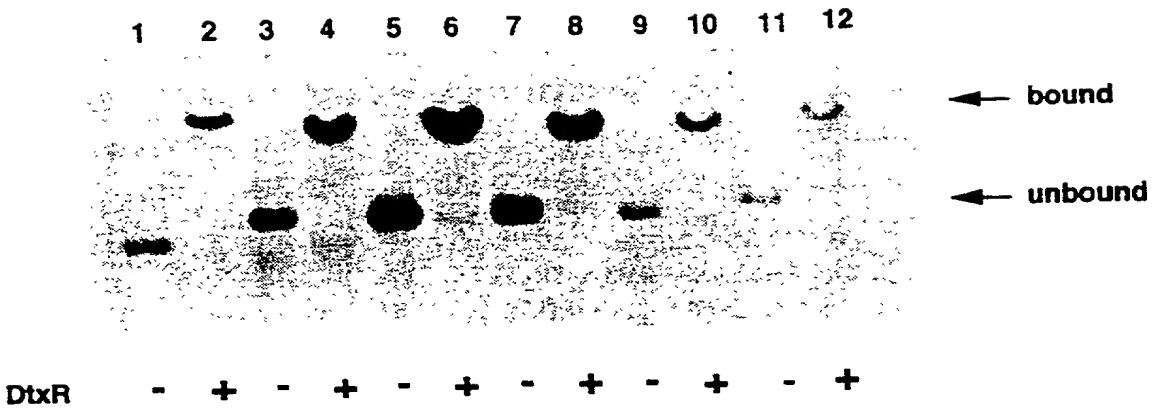


FIGURE 8

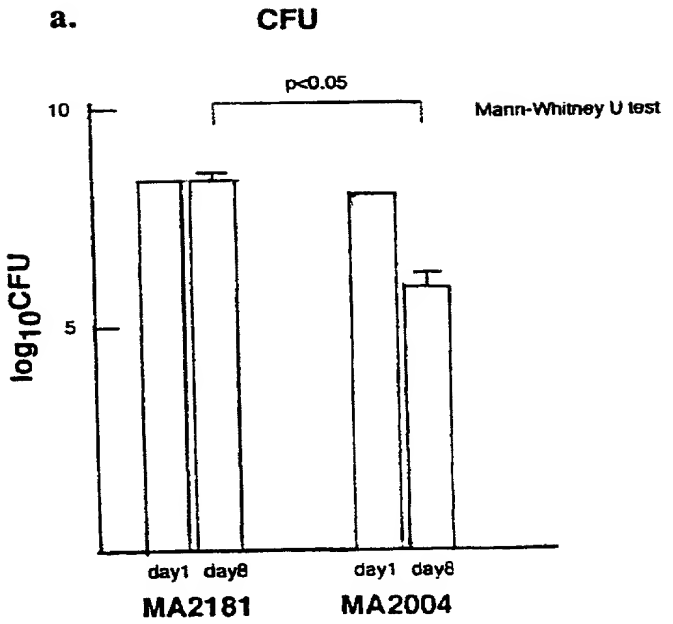


FIGURE 9A

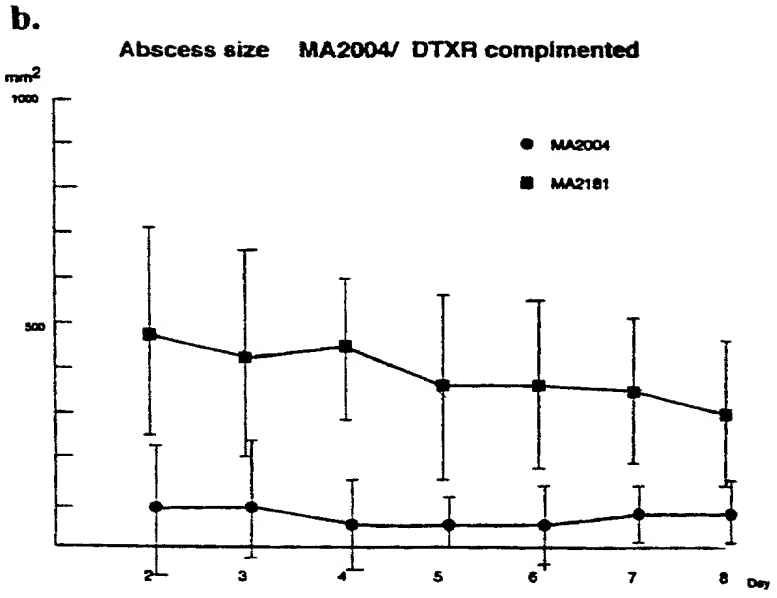


FIGURE 9B

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

ATTORNEY'S DOCKET NO.: AMSC 3.3-001

As a below-named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

VACCINE COMPOSITIONS the specification of which

☐ is attached hereto

☒ was filed on October 23, 2000 as United States Application Number or PCT International Application Number PCT/US00/29231 and was amended on __ ___ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (month, day, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐
LISTING OF FOREIGN APPLICATIONS CONTINUED ON PAGE 3 HEREOF ☐ YES ☐ NO			

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Application Number: 60/161,193 Filing Date: October 22, 1999

Application Number: 60/161,292 Filing Date: October 25, 1999

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Parent Application Serial Number:	Parent Filing Date:	Parent Patent No.:
U.S. Parent Application Serial Number.	Parent Filing Date:	Parent Patent No.:
PCT Parent Number:	Parent Filing Date:	

LISTING OF US APPLICATIONS CONTINUED ON PAGE 3 HEREOF: ☐ YES ☒ NO

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Customer Number 000530

DIRECT ALL CORRESPONDENCE TO: Customer No. 000530

DECLARATION -- Page 2

ATTORNEY DOCKET NO. AMSC 33-001

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

1-00 Full name of sole or first inventor (given name, family name): John R. MURPHY

Inventor's signature _____ Date 4/20/01

Residence: Boston, Massachusetts Citizenship US

Mailing Address: 130 Appleton Street, Boston MA 02116, U.S.A.

2-00 Full name of second joint inventor, if any (given name, family name) Edward O'LEAR

Second Inventor's signature _____ Date 6-21-01

Residence: Wolcott, Connecticut Citizenship: US

Mailing Address: 153 Spindle Hill Road, Wolcott, CT 06716, U.S.A.

3-00 Full name of third joint inventor, if any (given name, family name): Robert J. HARRISON

Third Inventor's signature _____ Date 6/20/01

Residence: Medfield, Massachusetts Citizenship: US

Mailing Address: 31 Green Street, Medfield, MA 02052

Full name of fourth joint inventor, if any (given name, family name):

Fourth Inventor's signature _____ Date _____

Residence: Citizenship.

Mailing Address:

Full name of fifth joint inventor (given name, family name):

Fifth Inventor's signature _____ Date _____

Residence: Citizenship:

Mailing Address:

Full name of sixth joint inventor, if any (given name, family name):

Sixth Inventor's signature _____ Date _____

Residence: Citizenship:

Mailing Address.

Full name of seventh joint inventor, if any (given name, family name):

Seventh Inventor's signature _____ Date _____

Residence: Citizenship:

Mailing Address:

Full name of eighth joint inventor, if any (given name, family name):

Eighth Inventor's signature _____ Date _____

Residence: Citizenship:

Mailing Address:

☐ Additional inventors are being named on separately numbered sheets attached hereto.

PATENT
AMSC 3.3-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of
John R. MURPHY et al.

International Application No.
PCT/US00/29231

International Filing Date:
23 October 2000

For: VACCINE COMPOSITIONS

X

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

STATEMENT ACCOMPANYING SEQUENCE LISTING

Sir:

Applicants enclose herewith the sequence listing in computer readable form (*i.e.*, a diskette) as well as a paper copy for the above referenced U.S. National application. The sequence listing does not include matter which goes beyond the content of the Application as filed and the information recorded on the diskette is identical to the written sequence listing.

Respectfully submitted,

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP

Gina Maldonado

Date: 6/15/01

600 South Avenue West
Westfield, NJ 07090
Telephone: (908)654-5000
Facsimile: (908)654-7866

SEQUENCE LISTING

<110> Murphy, John R.
O'Lear, Edward
Harrison, Robert J.

<120> Vaccine Compositions

<130> AMSC 3.3-001

<140> To be assigned
<141>

<150> PCT/US00/29231
<151> 2000-10-23

<160> 36

<170> PatentIn Ver. 2.1

<210> 1
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 1
accagatctg ccgaaaaact tcga 24

<210> 2
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 2
accagatctc cgcctttagt attta 25

<210> 3
<211> 27
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Native tox
operator

<400> 3
ataattagga tagctttacc taattat 27

<210> 4
<211> 19
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: DNA target site

<400> 4
gtaggttagg ctaacctat 19

<210> 5
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Consensus-binding sequence

<220>
<221> modified_base
<222> (1)..(25)
<223> "n" represents variable bases

<400> 5
ananttaggn tagnctannc tnnnn 25

<210> 6
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Variant DNA

<400> 6
twaggttags ctaacctwa 19

<210> 7
<211> 230
<212> PRT
<213> Mycobacterium tuberculosis

<400> 7
Met Asn Glu Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Asp Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
20 25 30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
35 40 45

```
Glu Arg Asp Gly Leu Leu Arg Val Ala Gly Asp Arg His Leu Glu Leu
     50                  55                  60

Thr Glu Lys Gly Arg Ala Leu Ala Ile Ala Val Met Arg Lys His Arg
 65                  70                  75                  80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu
                85                  90                  95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Asn Ser Glu Asp Val
            100                 105                 110

Glu Arg Arg Leu Val Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe
        115                 120                 125

Gly Asn Pro Ile Pro Gly Leu Val Glu Leu Gly Val Gly Pro Glu Pro
    130                 135                 140

Gly Ala Asp Asp Ala Asn Leu Val Arg Leu Thr Glu Leu Pro Ala Gly
145                 150                 155                 160

Ser Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
                165                 170                 175

Asp Ile Asp Leu Ile Thr Arg Leu Lys Asp Ala Gly Val Val Pro Asn
            180                 185                 190

Ala Arg Val Thr Val Glu Thr Thr Pro Gly Gly Gly Val Thr Ile Val
        195                 200                 205

Ile Pro Gly His Glu Asn Val Thr Leu Pro His Glu Met Ala His Ala
    210                 215                 220

Val Lys Val Glu Lys Val
225                 230
```

<210> 8
<211> 223
<212> PRT
<213> Cornyebacterium diptheriae

<400> 8

```
Met Lys Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
  1               5                  10                  15

Glu Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
            20                  25                  30

Arg Leu Glu Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
        35                  40                  45

Glu Arg Asp Gly Leu Val Val Val Ala Ser Asp Ser Leu Gln Met Thr
     50                  55                  60

Pro Thr Gly Arg Thr Leu Ala Thr Ala Val Met Arg Lys His Arg Leu
 65                  70                  75                  80
```

Ala Glu Arg Leu Leu Thr Asp Ile Ile Gly Leu Asp Ile Asn Lys Val
85 90 95

His Asp Glu Ala Cys Arg Trp Glu His Val Met Ser Asp Glu Val Glu
100 105 110

Arg Arg Leu Val Lys Val Lys Asp Val Ser Arg Ser Pro Phe Gly Asn
115 120 125

Pro Ile Pro Gly Leu Asp Glu Leu Gly Val Gly Asn Ser Asp Ala Ala
130 135 140

Ala Pro Gly Thr Arg Val Ile Asp Ala Ala Thr Ser Met Pro Arg Lys
145 150 155 160

Val Arg Ile Val Gln Ile Asn Glu Ile Phe Gln Val Glu Thr Asp Gln
165 170 175

Phe Gln Leu Leu Asp Ala Asp Ile Arg Val Gly Ser Glu Val Glu Ile
180 185 190

Val Asp Arg Asp Gly His Ile Thr Leu Ser His Asn Gly Lys Asp Val
195 200 205

Glu Leu Leu Asp Asp Leu Ala His Thr Ile Arg Ile Glu Glu Leu
210 215 220

<210> 9
<211> 174
<212> PRT
<213> Staphylococcus epidermitis

<400> 9
Met Thr Val Ser Cys Pro Pro Pro Ser Thr Ser Glu Arg Glu Glu Gln
1 5 10 15

Ala Arg Ala Leu Cys Leu Arg Leu Leu Thr Ala Arg Ser Arg Thr Arg
20 25 30

Ala Glu Leu Ala Gly Gln Leu Ala Lys Arg Gly Tyr Pro Glu Asp Ile
35 40 45

Gly Asn Arg Val Leu Asp Arg Leu Ala Ala Val Gly Leu Val Asp Asp
50 55 60

Thr Asp Phe Ala Glu Gln Trp Val Gln Ser Arg Arg Ala Asn Ala Ala
65 70 75 80

Lys Ser Lys Arg Ala Leu Ala Ala Glu Leu His Ala Lys Gly Val Asp
85 90 95

Asp Asp Val Ile Thr Thr Val Leu Gly Gly Ile Asp Ala Gly Ala Glu
100 105 110

Arg Gly Arg Ala Glu Lys Leu Val Arg Ala Arg Leu Arg Arg Glu Val

```
        115                 120                 125

Leu Ile Asp Asp Gly Thr Asp Glu Ala Arg Val Ser Arg Arg Leu Val
    130                 135                 140

Ala Met Leu Ala Arg Arg Gly Tyr Gly Gln Thr Leu Ala Cys Glu Val
145                 150                 155                 160

Val Ile Ala Glu Leu Ala Ala Glu Arg Glu Arg Arg Arg Val
                165                 170
```

<210> 10
<211> 225
<212> PRT
<213> Mycobacterium leprae

<400> 10

```
Met Asn Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
  1               5                  10                  15

Asp Leu Glu Glu Glu Gly Ile Val Thr Pro Leu Arg Ala Arg Ile Ala
             20                  25                  30

Glu Arg Pro Thr Val Ser Gln Thr Val Ser Arg Met Glu Arg Asp Gly
         35                  40                  45

Leu Leu Arg Val Ala Gly Asn Arg His Leu Glu Leu Thr Thr Lys Gly
     50                  55                  60

Arg Ala Met Ala Ile Ala Val Met Arg Lys His Arg Leu Ala Glu Arg
 65                  70                  75                  80

Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu Val His Ala Glu
                85                  90                  95

Ala Cys Arg Trp Glu His Val Met Ser Glu Asp Val Glu Arg Arg Leu
            100                 105                 110

Ile Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe Gly Asn Pro Ile
        115                 120                 125

Pro Gly Leu Leu Asp Leu Gly Ala Gly Pro Asp Ala Ser Ala Ala Asn
    130                 135                 140

Ala Lys Leu Val Arg Leu Thr Glu Leu Pro Ser Gly Ser Pro Val Ala
145                 150                 155                 160

Val Val Val Arg Gln Leu Thr Glu His Val Asp Asp Ile Asp Leu Ile
                165                 170                 175

Thr Arg Leu Lys Asp Thr Gly Val Val Pro Asn Ala Arg Val Thr Val
            180                 185                 190

Glu Thr Ser Pro Ala Gly Asn Val Ile Ile Ile Ile Pro Gly His Glu
        195                 200                 205
```

```
Asn Val Thr Leu Pro His Glu Met Ala His Ala Val Lys Val Glu Lys
    210                 215                 220

Val
225
```

<210> 11
<211> 230
<212> PRT
<213> Mycobacterium tuberculosis

<400> 11

```
Met Asn Glu Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
  1               5                  10                  15

Asp Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
             20                  25                  30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
         35                  40                  45

Glu Arg Asp Gly Leu Leu Arg Val Ala Gly Asp Arg His Leu Glu Leu
     50                  55                  60

Thr Glu Lys Gly Arg Ala Leu Ala Ile Ala Val Met Arg Lys His Arg
 65                  70                  75                  80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu
                 85                  90                  95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Asp Val
            100                 105                 110

Glu Arg Arg Leu Val Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe
        115                 120                 125

Gly Asn Pro Ile Pro Gly Leu Val Glu Leu Gly Val Gly Pro Glu Pro
    130                 135                 140

Gly Ala Asp Asp Ala Asn Leu Val Arg Leu Thr Glu Leu Pro Ala Gly
145                 150                 155                 160

Ser Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
                165                 170                 175

Asp Ile Asp Leu Ile Thr Arg Leu Lys Asp Ala Gly Val Val Pro Asn
            180                 185                 190

Ala Arg Val Thr Val Glu Thr Thr Pro Gly Gly Gly Val Thr Ile Val
        195                 200                 205

Ile Pro Gly His Glu Asn Val Thr Leu Pro His Glu Met Ala His Ala
    210                 215                 220

Val Lys Val Glu Lys Val
225                 230
```

<210> 12
<211> 233
<212> PRT
<213> Mycobacterium smegmatis

<400> 12
Met Asn Asp Leu Val Asp Thr Thr Glu Asn Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Asp Leu Glu Glu Glu Gly Val Val Pro Leu Arg Ala Arg Ile Ala Glu
20 25 30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
35 40 45

Glu Arg Asp Gly Leu Leu His Val Ala Gly Asp Arg His Leu Glu Leu
50 55 60

Thr Asp Lys Gly Arg Ala Leu Ala Val Ala Val Met Arg Lys His Arg
65 70 75 80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Asp
85 90 95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Glu Val
100 105 110

Glu Arg Arg Leu Val Gln Val Leu Glu Asn Pro Thr Thr Ser Pro Phe
115 120 125

Gly Asn Pro Ile Pro Gly Leu Thr Glu Leu Ala Val Thr Pro Gly Val
130 135 140

Asn Thr Glu Asp Val Ser Leu Val Arg Leu Thr Glu Leu Pro Val Gly
145 150 155 160

Met Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
165 170 175

Asp Thr Asp Leu Ile Gly Arg Leu Lys Glu Ala Gly Val Val Pro Asn
180 185 190

Ala Arg Val Thr Val Glu Ala Asn Asn Asn Gly Gly Val Met Ile Val
195 200 205

Ile Pro Gly His Glu Gln Val Glu Leu Pro His His Met Ala His Ala
210 215 220

Val Lys Val Glu Lys Val Glu Lys Val
225 230

<210> 13
<211> 174
<212> PRT

<213> Mycobacterium tuberculosis

<400> 13

```
Met Thr Val Ser Cys Pro Pro Pro Ser Thr Ser Glu Arg Glu Glu Gln
  1               5                  10                  15

Ala Arg Ala Leu Cys Leu Arg Leu Leu Thr Ala Arg Ser Arg Thr Arg
             20                  25                  30

Ala Glu Leu Ala Gly Gln Leu Ala Lys Arg Gly Tyr Pro Glu Asp Ile
         35                  40                  45

Gly Asn Arg Val Leu Asp Arg Leu Ala Ala Val Gly Leu Val Asp Asp
     50                  55                  60

Thr Asp Phe Ala Glu Gln Trp Val Gln Ser Arg Arg Ala Asn Ala Ala
 65                  70                  75                  80

Lys Ser Lys Arg Ala Leu Ala Ala Glu Leu His Ala Lys Gly Val Asp
                 85                  90                  95

Asp Asp Val Ile Thr Thr Val Leu Gly Gly Ile Asp Ala Gly Ala Glu
            100                 105                 110

Arg Gly Arg Ala Glu Lys Leu Val Arg Ala Arg Leu Arg Arg Glu Val
        115                 120                 125

Leu Ile Asp Asp Gly Thr Asp Glu Ala Arg Val Ser Arg Arg Leu Val
    130                 135                 140

Ala Met Leu Ala Arg Arg Gly Tyr Gly Gln Thr Leu Ala Cys Glu Val
145                 150                 155                 160

Val Ile Ala Glu Leu Ala Ala Glu Arg Glu Arg Arg Arg Val
                165                 170
```

<210> 14
<211> 228
<212> PRT
<213> Brevibacterium lactofermentum

<400> 14

```
Met Lys Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
  1               5                  10                  15

Glu Leu Glu Glu Glu Gly Ile Val Pro Leu Arg Ala Arg Ile Ala Glu
             20                  25                  30

Arg Leu Glu Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
         35                  40                  45

Glu Arg Asp Gly Leu Val His Val Ser Pro Asp Arg Ser Leu Glu Met
     50                  55                  60

Thr Pro Glu Gly Arg Ser Leu Ala Ile Ala Val Met Arg Asn Asp Arg
 65                  70                  75                  80
```

Leu Ala Glu Arg Leu Leu Thr Asp Ile Ile Gly Leu Asp Ile His Lys
85 90 95

Val His Asp Glu Ala Cys Arg Trp Glu His Val Met Ser Asp Glu Val
100 105 110

Glu Arg Arg Leu Val Glu Val Leu Asp Asp Val His Arg Ser Pro Phe
115 120 125

Gly Asn Pro Ile Pro Gly Leu Gly Glu Ile Gly Leu Asp Gln Ala Asp
130 135 140

Glu Pro Asp Ser Gly Val Arg Ala Ile Asp Leu Pro Leu Gly Glu Asn
145 150 155 160

Leu Lys Ala Arg Ile Val Gln Leu Asn Glu Ile Leu Gln Val Asp Leu
165 170 175

Glu Gln Phe Gln Ala Leu Thr Asp Ala Gly Val Glu Ile Gly Thr Glu
180 185 190

Val Asp Ile Ile Asn Glu Gln Gly Arg Val Val Ile Thr His Asn Gly
195 200 205

Ser Ser Val Glu Leu Ile Asp Asp Leu Ala His Ala Val Arg Val Glu
210 215 220

Lys Val Glu Gly
225

<210> 15
<211> 226
<212> PRT
<213> Corynebacterium diphtheriae

<400> 15
Met Lys Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Glu Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
20 25 30

Arg Leu Glu Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
35 40 45

Glu Arg Asp Gly Leu Val Val Val Ala Ser Asp Arg Ser Leu Gln Met
50 55 60

Thr Pro Thr Gly Arg Thr Leu Ala Thr Ala Val Met Arg Lys His Arg
65 70 75 80

Leu Ala Glu Arg Leu Leu Thr Asp Ile Ile Gly Leu Asp Ile Asn Lys
85 90 95

Val His Asp Glu Ala Cys Arg Trp Glu His Val Met Ser Asp Glu Val

100 105 110

Glu Arg Arg Leu Val Lys Val Leu Lys Asp Val Ser Arg Ser Pro Phe
115 120 125

Gly Asn Pro Ile Pro Gly Leu Asp Glu Leu Gly Val Gly Asn Ser Asp
130 135 140

Ala Ala Ala Pro Gly Thr Arg Val Ile Asp Ala Ala Thr Ser Met Pro
145 150 155 160

Arg Lys Val Arg Ile Val Gln Ile Asn Glu Ile Phe Gln Val Glu Thr
165 170 175

Asp Gln Phe Thr Gln Leu Leu Asp Ala Asp Ile Arg Val Gly Ser Glu
180 185 190

Val Glu Ile Val Asp Arg Asp Gly His Ile Thr Leu Ser His Asn Gly
195 200 205

Lys Asp Val Glu Leu Leu Asp Asp Leu Ala His Thr Ile Arg Ile Glu
210 215 220

Glu Leu
225

<210> 16
<211> 230
<212> PRT
<213> Mycobacterium tuberculosis

<400> 16
Met Asn Glu Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Asp Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
20 25 30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
35 40 45

Glu Arg Asp Gly Leu Leu Arg Val Ala Gly Asp Arg His Leu Glu Leu
50 55 60

Thr Glu Lys Gly Arg Ala Leu Ala Ile Ala Val Met Arg Lys His Arg
65 70 75 80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu
85 90 95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Asp Val
100 105 110

Glu Arg Arg Leu Val Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe
115 120 125

```
Gly Asn Pro Ile Pro Gly Leu Val Glu Leu Gly Val Gly Pro Glu Pro
    130                 135                 140

Gly Ala Asp Asp Ala Asn Leu Val Arg Leu Thr Glu Leu Pro Ala Gly
145                 150                 155                 160

Ser Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
                165                 170                 175

Asp Ile Asp Leu Ile Thr Arg Leu Lys Asp Ala Gly Val Val Pro Asn
            180                 185                 190

Ala Arg Val Thr Val Glu Thr Thr Pro Gly Gly Gly Val Thr Ile Val
        195                 200                 205

Ile Pro Gly His Glu Asn Val Thr Leu Pro His Glu Met Ala His Ala
    210                 215                 220

Val Lys Val Glu Lys Val
225                 230


<210> 17
<211> 235
<212> PRT
<213> Mycobacterium smegmatis

<400> 17
Met Asn Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
  1               5                  10                  15

Asp Leu Glu Glu Glu Gly Val Val Pro Leu Arg Ala Arg Ile Ala Glu
            20                  25                  30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
        35                  40                  45

Glu Arg Asp Gly Leu Leu His Val Ala Gly Asp Arg His Leu Glu Leu
    50                  55                  60

Thr Asp Lys Gly Arg Ala Leu Ala Val Ala Val Met Arg Lys His Arg
65                  70                  75                  80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Leu Pro Trp Glu Asp Gly
                85                  90                  95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Glu Val
            100                 105                 110

Glu Arg Arg Leu Val Gln Val Leu Glu Asn Pro Thr Thr Ser Pro Phe
        115                 120                 125

Gly Asn Pro Ile Pro Gly Leu Thr Glu Leu Ala Val Thr Pro Gly Val
    130                 135                 140

Asn Thr Glu Asp Val Ser Leu Val Arg Leu Thr Glu Leu Pro Val Gly
145                 150                 155                 160
```

Met Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
165 170 175

Asp Thr Asp Leu Ile Gly Arg Leu Lys Glu Ala Gly Val Val Pro Asn
180 185 190

Ala Arg Val Thr Val Glu Ala Asn Asn Asn Gly Gly Val Met Ile Val
195 200 205

Ile Pro Gly His Glu Gln Val Glu Leu Pro His His Met Ala His Ala
210 215 220

Val Lys Lys Lys Val Glu Lys Val Glu Lys Val
225 230 235

<210> 18
<211> 225
<212> PRT
<213> Mycobacterium leprae

<400> 18
Met Asn Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Asp Leu Glu Glu Glu Gly Ile Val Thr Pro Leu Arg Ala Arg Ile Ala
20 25 30

Glu Arg Pro Thr Val Ser Gln Thr Val Ser Arg Met Glu Arg Asp Gly
35 40 45

Leu Leu Arg Val Ala Gly Asn Arg His Leu Glu Leu Thr Thr Lys Gly
50 55 60

Arg Ala Met Ala Ile Ala Val Met Arg Lys His Arg Leu Ala Glu Arg
65 70 75 80

Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu Val His Ala Glu
85 90 95

Ala Cys Arg Trp Glu His Val Met Ser Glu Asp Val Glu Arg Arg Leu
100 105 110

Ile Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe Gly Asn Pro Ile
115 120 125

Pro Gly Leu Leu Asp Leu Gly Ala Gly Pro Asp Ala Ser Ala Ala Asn
130 135 140

Ala Lys Leu Val Arg Leu Thr Glu Leu Pro Ser Gly Ser Pro Val Ala
145 150 155 160

Val Val Val Arg Gln Leu Thr Glu His Val Asp Asp Ile Asp Leu Ile
165 170 175

Thr Arg Leu Lys Asp Thr Gly Val Val Pro Asn Ala Arg Val Thr Val

```
            180                 185                 190

Glu Thr Ser Pro Ala Gly Asn Val Ile Ile Ile Ile Pro Gly His Glu
        195                 200                 205

Asn Val Thr Leu Pro His Glu Met Ala His Ala Val Lys Val Glu Lys
    210                 215                 220

Val
225
```

<210> 19
<211> 230
<212> PRT
<213> Streptomyces lividans

<400> 19

```
Met Ser Gly Leu Ile Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Leu
  1               5                  10                  15

Glu Leu Glu Glu Glu Gly Val Val Pro Met Arg Ala Arg Ile Ala Glu
             20                  25                  30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
         35                  40                  45

Glu Arg Asp Gly Leu Val Ser Val Ala Ala Asp Arg His Leu Glu Leu
     50                  55                  60

Thr Asp Glu Gly Arg Arg Leu Ala Thr Arg Val Met Arg Lys His Arg
 65                  70                  75                  80

Leu Ala Glu Cys Leu Leu Val Asp Val Ile Gly Leu Glu Trp Glu Gln
                 85                  90                  95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Ala Val
            100                 105                 110

Glu Arg Arg Val Leu Glu Leu Leu Arg His Pro Thr Glu Ser Pro Tyr
        115                 120                 125

Gly Asn Pro Ile Pro Gly Leu Glu Glu Leu Gly Glu Thr Asp Gly Ala
    130                 135                 140

Asp Pro Phe Leu Asp Glu Gly Met Val Ser Leu Ala Asp Leu Asp Pro
145                 150                 155                 160

Gly Gln Glu Gly Lys Thr Val Val Val Arg Arg Ile Gly Glu Pro Ile
                165                 170                 175

Gln Thr Asp Ala Gln Leu Met Tyr Thr Leu Arg Arg Ala Gly Val Gln
            180                 185                 190

Pro Gly Ser Val Val Ser Val Thr Glu Ser Ala Gly Gly Val Leu Val
        195                 200                 205
```

```
Gly Ser Gly Gly Glu Ala Ala Glu Leu Glu Ala Asp Thr Ala Ser His
    210                 215                 220

Val Phe Val Ala Lys Arg
225                 230


<210> 20
<211> 215
<212> PRT
<213> Staphylococcus epidermidis

<400> 20
Met Leu Thr Glu Glu Lys Glu Asp Tyr Leu Lys Ala Ile Leu Thr Asn
  1               5                  10                  15

Asp Gly Asp Val Ser Phe Val Ser Asn Lys Lys Leu Ser Gln Phe Leu
             20                  25                  30

Asn Ile Lys Pro Pro Ser Val Ser Glu Met Val Gly Arg Leu Glu Lys
         35                  40                  45

Glu Gly Tyr Val Glu Thr Lys His Tyr Lys Gly Ala Arg Leu Thr Glu
     50                  55                  60

Glu Gly Leu Lys Gln Thr Leu Asp Ile Ile Lys Arg His Arg Leu Leu
 65                  70                  75                  80

Arg Leu Phe Leu Ile Glu Ile Leu Gln Tyr Asn Trp Glu Glu Val His
                 85                  90                  95

Gln Glu Ala Glu Ile Leu Glu His Arg Ile Ser Asp Leu Phe Val Glu
            100                 105                 110

Arg Leu Asp Lys Ile Leu Asn Phe Pro Lys Thr Cys Pro His Gly Gly
        115                 120                 125

Val Ile Pro Arg Gly Asn Ser Asp Ala Ala Ala Pro Gly Thr Ser Ile
    130                 135                 140

Leu Asn Phe Glu Pro Gly Glu Arg Val Thr Val Arg Arg Val Arg Arg
145                 150                 155                 160

Asp Lys Thr Glu Leu Leu Val Tyr Leu Ser Ser Lys Asp Ile Tyr Ile
                165                 170                 175

Gly Asn Thr Val Glu Ile Val Ser Lys Asp Asp Thr Asn Lys Val Ile
            180                 185                 190

Ile Leu Lys Arg Asn Asp Ile Val Thr Ile Leu Ser Tyr Glu Asn Ala
        195                 200                 205

Met Asn Ile Phe Ala Glu Lys
    210                 215


<210> 21
```

<211> 213
<212> PRT
<213> Staphylococcus aureus

<400> 21
Met Leu Thr Glu Glu Lys Glu Asp Tyr Leu Lys Ala Ile Leu Thr Asn
1 5 10 15

Asn Gly Asp Lys Asn Phe Val Thr Asn Lys Ile Leu Ser Gln Phe Leu
20 25 30

Asn Ile Lys Pro Pro Ser Val Ser Glu Met Val Gly Arg Leu Glu Lys
35 40 45

Ala Gly Tyr Val Glu Thr Lys Pro Tyr Lys Gly Val Arg Leu Thr Glu
50 55 60

Asp Gly Leu Thr His Thr Leu Asp Ile Ile Arg His Arg Leu Leu Glu
65 70 75 80

Leu Phe Leu Ile Glu Ile Leu Lys Tyr Asn Trp Glu Glu Val His Gln
85 90 95

Glu Ala Glu Ile Leu Glu His Arg Ile Ser Asp Leu Phe Val Glu Arg
100 105 110

Leu Asp Ser Leu Leu Asn Phe Pro Glu Thr Cys Pro His Gly Gly Val
115 120 125

Ile Pro Arg Asn Asn Glu Tyr Lys Glu Lys Tyr Ile Thr Thr Ile Leu
130 135 140

Asn Tyr Glu Pro Gly Asp Ile Val Thr Ile Lys Arg Val Arg Asp Lys
145 150 155 160

Thr Asp Leu Leu Ile Tyr Leu Ser Ser Lys Asp Ile Ser Ile Gly Asn
165 170 175

Glu Val Glu Ile Val Ser Lys Asp Glu Met Asn Lys Val Ile Ile Ile
180 185 190

Lys Arg Asn Asp Asn Val Ile Ile Val Ser Tyr Glu Asn Ala Met Asn
195 200 205

Met Phe Ala Glu Lys
210

<210> 22
<211> 222
<212> PRT
<213> Enterococcus faecalis

<400> 22
Met Thr Pro Asn Arg Glu Asp Tyr Leu Lys Leu Ile Phe Glu Leu Gly
1 5 10 15

```
Gly Asp Glu Val Lys Val Asn Asn Lys Gln Ile Val Ser Gly Leu Asp
            20                  25                  30

Val Ser Ala Ala Ser Val Ser Glu Met Ile Ser Lys Leu Val Lys Glu
        35                  40                  45

Asp Leu Val Glu His Ser Pro Tyr Gln Gly Val Gln Leu Thr Glu Lys
    50                  55                  60

Gly Leu Lys Lys Ala Ser Thr Leu Ile Arg Lys His Arg Ile Trp Glu
65                  70                  75                  80

Val Phe Leu Val Glu His Leu Asn Tyr Thr Trp Asn Asp Val His Glu
                85                  90                  95

Glu Ala Glu Val Leu Glu His Val Thr Ser Gln Thr Leu Val Asn Arg
            100                 105                 110

Leu Ala Asp Tyr Leu Asn His Pro Glu Phe Cys Pro His Gly Gly Val
        115                 120                 125

Ile Pro Glu Asp Asn Gln Pro Ile His Glu Glu Lys Arg Gln Thr Leu
    130                 135                 140

Thr Asp Tyr Pro Val Gly Thr Lys Ile Arg Ile Ala Arg Val Leu Asp
145                 150                 155                 160

Glu Lys Glu Leu Leu Asp Tyr Leu Val Ser Ile Asp Leu Asn Ile Gln
                165                 170                 175

Glu Glu Tyr Thr Ile Lys Glu Ile Ala Ala Tyr Glu Gly Pro Ile Thr
            180                 185                 190

Ile Tyr Asn Glu Asn Lys Glu Leu Ser Val Ser Phe Lys Ala Ala Asn
        195                 200                 205

Thr Ile Phe Val Glu Pro Leu Ile Arg Glu Ser Glu Glu Asn
    210                 215                 220
```

<210> 23
<211> 215
<212> PRT
<213> Streptococcus gordonii

<400> 23

```
Met Thr Pro Asn Lys Glu Asp Tyr Leu Lys Cys Leu Tyr Glu Leu Gly
  1               5                  10                  15

Thr Arg His Asn Lys Ile Thr Asn Lys Glu Ile Ala Gly Leu Met Gln
            20                  25                  30

Val Ser Pro Pro Ala Val Thr Glu Met Met Lys Lys Leu Leu Ala Glu
        35                  40                  45

Glu Leu Leu Ile Lys Asp Lys Lys Ala Gly Tyr Leu Leu Thr Asp Leu
    50                  55                  60
```

Gly Leu Lys Leu Val Ser Asp Leu Tyr Arg Lys His Arg Leu Ile Glu
65 70 75 80

Val Phe Leu Val His His Leu Gly Tyr Thr Thr Glu Glu Ile His Glu
85 90 95

Glu Ala Glu Val Leu Glu His Thr Val Ser Asp His Phe Val Glu Arg
100 105 110

Leu Asp Gln Leu Leu Asp Tyr Pro Lys Ala Cys Pro His Gly Gly Thr
115 120 125

Ile Pro Ala Lys Gly Glu Leu Leu Val Glu Lys His Lys Leu Thr Leu
130 135 140

Glu Glu Ala Lys Glu Lys Gly Asp Tyr Ile Leu Ala Arg Val His Asp
145 150 155 160

Asn Phe Asp Leu Leu Thr Tyr Leu Glu Arg Asn Gly Leu Gln Val Gly
165 170 175

Lys Thr Ile Arg Phe Leu Gly Tyr Asp Asp Phe Ser His Leu Tyr Ser
180 185 190

Leu Glu Val Asp Gly Gln Glu Ile Gln Leu Ala Gln Pro Ile Ala Gln
195 200 205

Gln Ile Tyr Val Glu Lys Ile
210 215

<210> 24
<211> 217
<212> PRT
<213> Streptococcus mutans

<400> 24
Met Thr Pro Asn Lys Glu Asp Tyr Leu Lys Ile Ile Tyr Glu Leu Ser
1 5 10 15

Glu Arg Asp Glu Lys Ile Ser Asn Lys Gln Ile Ala Glu Lys Met Ser
20 25 30

Val Ser Ala Pro Ala Val Ser Glu Met Val Lys Lys Leu Leu Leu Glu
35 40 45

Asp Leu Val Leu Lys Asp Lys Gln Ala Gly Tyr Leu Leu Thr Lys Lys
50 55 60

Gly Gln Ile Leu Ala Ser Ser Leu Tyr Arg Lys His Arg Leu Ile Glu
65 70 75 80

Val Phe Leu Met Asn His Leu Asn Tyr Thr Ala Asp Glu Ile His Glu
85 90 95

Glu Ala Glu Val Leu Glu His Thr Val Ser Asp Val Phe Val Glu Arg

                100   105   110

Leu Asp Lys Phe Leu Asn Tyr Pro Lys Val Cys Pro His Gly Gly Thr
        115   120   125

Ile Pro Gly His Gly Gln Pro Leu Val Glu Arg Tyr Arg Thr Thr Leu
    130   135   140

Lys Gly Val Thr Glu Met Gly Val Tyr Leu Leu Lys Arg Val Gln Asp
145   150   155   160

Asn Phe Gln Leu Leu Lys Tyr Met Glu Gln His His Leu Lys Ile Gly
                165   170   175

Asp Glu Leu Arg Leu Leu Glu Tyr Asp Ala Phe Ala Gly Ala Tyr Thr
            180   185   190

Ile Glu Lys Asp Gly Glu Gln Leu Gln Val Thr Ser Ala Val Ala Ser
        195   200   205

Gln Ile Tyr Ile Glu Lys Lys Ala Tyr
    210   215

<210> 25
<211> 216
<212> PRT
<213> Streptococcus pneumoniae

<400> 25
Met Thr Pro Asn Lys Glu Asp Tyr Leu Lys Cys Ile Tyr Glu Ile Gly
 1   5   10   15

Ile Asp Leu His Lys Ile Thr Asn Lys Glu Ile Ala Ala Arg Met Gln
            20   25   30

Val Ser Pro Pro Ala Val Thr Glu Met Ile Lys Arg Met Lys Ser Glu
        35   40   45

Asn Leu Ile Leu Lys Asp Lys Glu Cys Gly Tyr Leu Leu Thr Asp Leu
    50   55   60

Gly Leu Lys Leu Val Ser Glu Leu Tyr Arg Lys His Arg Leu Ile Glu
65   70   75   80

Val Phe Leu Val His His Leu Asp Tyr Thr Ser Asp Gln Ile His Glu
                85   90   95

Glu Ala Glu Val Leu Glu His Thr Val Ser Asp Leu Phe Val Glu Arg
            100   105   110

Leu Asp Lys Leu Leu Gly Phe Pro Lys Thr Cys Pro His Gly Gly Thr
        115   120   125

Ile Pro Ala Lys Gly Glu Leu Leu Val Glu Ile Asn Asn Leu Pro Leu
    130   135   140

```
Ala Asp Ile Lys Glu Ala Gly Ala Tyr Arg Leu Thr Arg Val His Asp
145                 150                 155                 160

Ser Phe Asp Ile Leu His Tyr Leu Asp Lys His Ser Leu His Ile Gly
                165                 170                 175

Asp Gln Leu Gln Val Lys Gln Phe Asp Gly Phe Ser Asn Thr Phe Thr
            180                 185                 190

Ile Leu Ser Asn Asp Glu Asp Leu Gln Val Asn Met Asp Ile Ala Lys
        195                 200                 205

Gln Leu Tyr Val Glu Lys Ile Asn
    210                 215
```


<210> 26
<211> 216
<212> PRT
<213> Streptococcus pyogenes

<400> 26

```
Met Thr Pro Asn Lys Glu Asp Tyr Leu Lys Cys Ile Tyr Glu Ile Gly
  1               5                  10                  15

Ile Asp Leu His Lys Ile Thr Asn Lys Glu Ile Ala Ala Arg Met Gln
            20                  25                  30

Val Ser Pro Pro Ala Val Thr Glu Met Ile Lys Arg Met Lys Ser Glu
        35                  40                  45

Asn Leu Ile Leu Lys Asp Lys Glu Cys Gly Tyr Leu Leu Thr Asp Leu
    50                  55                  60

Gly Leu Lys Leu Val Ser Glu Leu Tyr Arg Lys His Arg Leu Ile Glu
65                  70                  75                  80

Val Phe Leu Val His His Leu Asp Tyr Thr Ser Asp Gln Ile His Glu
                85                  90                  95

Glu Ala Glu Val Leu Glu His Thr Val Ser Asp Leu Phe Val Glu Arg
            100                 105                 110

Leu Asp Lys Leu Leu Gly Phe Pro Lys Thr Cys Pro His Gly Gly Thr
        115                 120                 125

Ile Pro Ala Lys Gly Glu Leu Leu Val Glu Ile Asn Asn Leu Pro Leu
    130                 135                 140

Ala Asp Ile Lys Glu Ala Gly Ala Tyr Arg Leu Thr Arg Val His Asp
145                 150                 155                 160

Ser Phe Asp Ile Leu His Tyr Leu Asp Lys His Ser Leu His Ile Gly
                165                 170                 175

Asp Gln Leu Gln Val Lys Gln Phe Asp Gly Phe Ser Asn Thr Phe Thr
            180                 185                 190
```

Ile Leu Ser Asn Asp Glu Asp Leu Gln Val Asn Met Asp Ile Ala Lys
195 200 205

Gln Leu Tyr Val Glu Lys Ile Asn
210 215

<210> 27
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Consensus sequence

<400> 27
gtaggttagg ctaacctat 19

<210> 28
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Consensus sequence

<400> 28
ttaggttagg ctaacctaa 19

<210> 29
<211> 19
<212> DNA
<213> Cornyebacterium diptheriae

<400> 29
ttaggatagc tttacctaa 19

<210> 30
<211> 19
<212> DNA
<213> Streptomyces pilosus

<400> 30
ttaggttagg ctcacctaa 19

<210> 31
<211> 19
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: 16S ribosomal RNA

<400> 31
ccagggtatc taatcctgt 19

<210> 32
<211> 19
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: 19 kDa antigen

<400> 32
gcaggccagt gaaacctgt 19

<210> 33
<211> 20
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: htrA homologue

<400> 33
acaggtggtg ctcaaccacg 20

<210> 34
<211> 20
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: phoP homologue

<400> 34
gaaggtaacg ttcaaccaat 20

<210> 35
<211> 20
<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: adhB homologue

<400> 35
gcaggtgacc gtcaaccgat 20

<210> 36
<211> 19

<212> DNA
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: narG homologue

<400> 36
gaaggtcaac caaacaaga 19